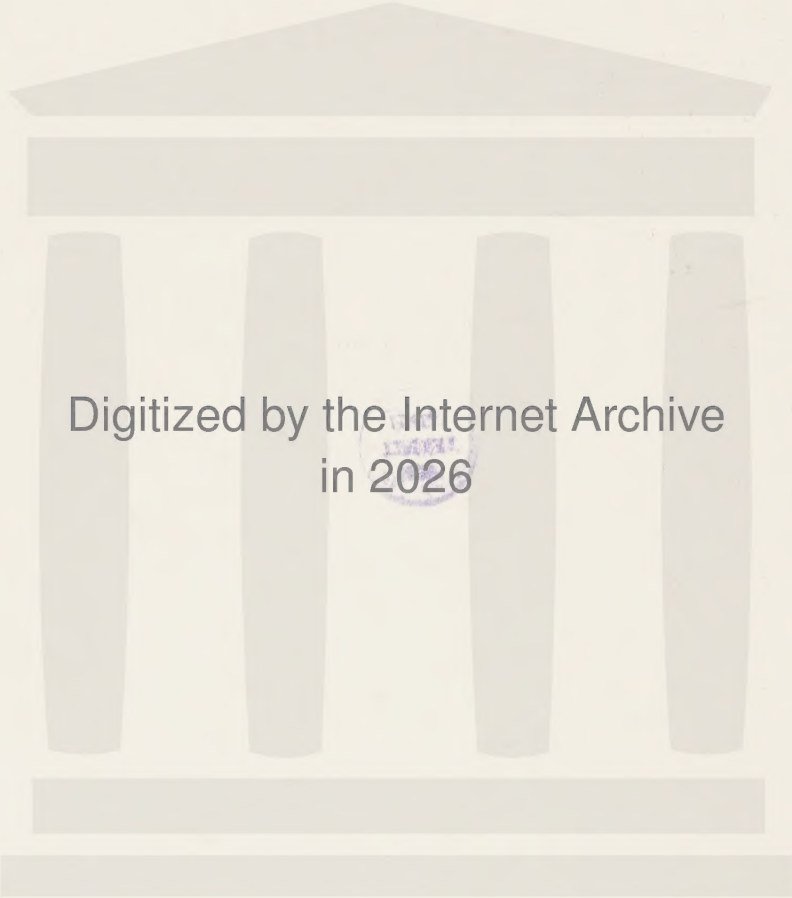


THE GABA NEUROTRANSMITTER SYSTEM IN THE RETINA

Elisabet Agardh

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av

Elisabet Agardh

Leg. läkare

Ld

Organisation LUND UNIVERSITY Department of Ophthalmology University Hospital S-221 85 Lund Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue Jan. 11 1985	
		CODEN: LUNEDW/ (MEVL-1006)/1-39 (1985)	
Author(s) Elisabet Agardh		Sponsoring organization	
Title and subtitle The GABA neurotransmitter System in the Retina			
Abstract (³H)Isoguvacine was shown to be useful in morphological studies of presumed GABA neurons. In all species examined, (³H)isoguvacine labelled neurons in a way similar to that of (³H)GABA, but in contrast, (³H)isoguvacine was not taken up into glial cells. For the first time, it has been possible to visualize presumed GABA neurons in some mammals, e.g. man, where in previous studies the glial labelling with (³H)GABA was too pronounced. Potassium caused an increased release of (³H)GABA from the rabbit retina, which was not Ca⁺⁺-dependent. The results indicate a non-vesicular release of neurotransmitter. The effects of some other putative neurotransmitters, glutamate, aspartate, glycine, and GABA were also examined. None of them affected the release of (³H)GABA. Most likely, neurons with these transmitters do not control the GABA neurons in the rabbit retina. In intact chick retina, the GABA antagonists, bicuculline and picrotoxin, increased the release of (³H)acetylcholine. The results indicate that the cholinergic neurons are tonically inhibited by endogenous GABA. In a culture system, the inhibitory effects of GABA could be demonstrated at the level of one single neuron. The results indicate that GABA mediates its inhibitory effect via receptors located on the cholinergic neurons.			
Key words Neurotransmission, GABA, retina, acetylcholine, release, autoradiography, electrophysiology, isoguvacine			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		Language English	
ISSN and key title		ISBN	
Recipient's notes		Number of pages 39	Price
		Security classification	

Distribution by (name and address) Dr Elisabet Agardh
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Lund 1984

This thesis is based on the following papers:

- I. Agardh, E. and Ehinger, B.: (^3H)Muscimol, (^3H)nipecotic acid and (^3H)isoguvacine as autoradiographic markers for GABA neurotransmission. *J. Neural Transmission* 54:1-18, 1982.
- II. Agardh, E. and Ehinger, B.: Retinal GABA neuron labelling with (^3H)isoguvacine in different species. *Exp. Eye Res.* 36:215-229, 1983.
- III. Agardh, E. and Ehinger, B.: The uptake of the GABA agonist, (^3H)isoguvacine, by the isolated retina of the rabbit. *Neurochem. Int.* 5:779-783, 1983.
- IV. Agardh, E. and Bauer, B.: The release of (^3H)GABA from the rabbit retina. *Acta Physiol. Scand.* 122:85-92, 1984.
- V. Agardh, E.: GABA inhibits the spontaneous release of (^3H)acetylcholine from the chick retina. Submitted for publication.
- VI. Agardh, E., Yeh, H. H., Herrmann, R. and Puro, D. G.: GABA-mediated inhibition at cholinergic synapses formed by cultured retinal neurons. *Brain Res.* In press, 1984.

The papers will be referred to in the text by their Roman numerals.

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INTRODUCTION

The retina is a specialized structure of the central nervous system, which responds to visual stimuli of many different kinds. The signal starts in the photoreceptor layer, is transmitted at junctions in the outer plexiform layer to the bipolar cells, is passed from the bipolar cells via junctions in the inner plexiform layer to the ganglion cells and is sent further through ganglion cell axons to the brain. The signal is influenced by horizontal cells at the level of the outer plexiform layer and by amacrine cells at the level of the inner plexiform layer. In addition, interplexiform cells form feed-back loops from the inner to the outer plexiform layer. For a recent review, see Ehinger (1982).

The signal is electrically transmitted between the cells at gap junctions, or with the aid of chemical neurotransmitters at other junctions. Numerous substances have been proposed as neurotransmitters: acetylcholine, amino acids, indoleamines, catecholamines, and several peptides (Ehinger 1982, Brecha 1983). With a few exceptions, these putative transmitters have been localized to one group of neurons in the inner part of the retina, the amacrine cells.

Biochemical, histochemical, and pharmacological studies suggest that GABA may be a neurotransmitter in the retina. This presumption, while not yet definitively proven, is based on the following observations.

GABA Biochemistry

GABA is present in the retina (Pasantes-Morales et al. 1972, 1973, Oraedu et al. 1980), and is most concentrated in



the inner part, where amacrine neurons and ganglion cells are located. GABA is also present in the inner plexiform layer where these cells form synapses (Kuriyama et al. 1968, Berger et al. 1977, Kennedy et al. 1977, Morjaria and Voaden 1979, Dick and Lowry 1984). The rate-limiting synthesizing enzyme, glutamate decarboxylase (GAD, EC 4.1.1.15), and the degrading enzyme aminobutyrate amino transaminase (GABA-T, EC 2.6.1.1.19) have been demonstrated in the retina (GAD: Kuriyama et al. 1968, Lam 1975, Sarthy and Lam 1979, Brandon et al. 1979, 1980, Vaughn et al. 1981, Dick and Lowry 1984, Pusateri et al. 1984, GABA-T: Starr 1973, Dick and Lowry 1984, Pusateri et al. 1984).

In addition, high affinity GABA binding has been demonstrated in the goat, sheep, and cow (Enna and Snyder 1976, Redburn et al. 1979, 1980, Redburn and Mitchell 1981) and a high affinity uptake of GABA has also been shown in a number of species (Starr and Voaden 1972, Goodchild and Neal 1973, Neal 1976).

GABA Histochemistry

To identify the cells that presumably use GABA as a neurotransmitter, the uptake pattern of (^3H)GABA has been studied, as well as the localization of GAD immunoreactivity. In some mammals, (^3H)GABA is accumulated mainly by amacrine cells, by the inner plexiform layer where the amacrine neurons have their synaptic contacts, and by cells in the ganglion cell layer. In other mammals, (^3H)GABA is also taken up by glial cells (Neal and Iversen 1972, Bruun and Ehinger 1974, Marshall and Voaden 1974a, 1975, Ehinger 1977, Brandon et al. 1979). In bird and fish, (^3H)GABA is accumulated by

amacrine neurons, by the inner plexiform layer, and by ganglion cells. In addition, (^3H)GABA is in these species accumulated in a different type of retinal neurons, the horizontal cells, and in the outer plexiform layer, where these cells extend their processes (Lam and Steinman 1971, Marshall and Voaden 1974b, Marc et al. 1978). Glial cells become only weakly labelled in these species. The GAD immunoreactivity pattern in general matches the (^3H)GABA uptake pattern (Brandon et al. 1979, Stell 1984). At the ultrastructural level, the colocalization of GAD and of (^3H)GABA uptake is still controversial (Zucker et al. 1984, Stell 1984).

GABA Release Studies

Release studies are important in establishing a putative neurotransmitter as a true neurotransmitter. Potassium-induced release of radioactivity has been demonstrated from rabbit retinal synaptosomes preloaded with (^3H)GABA or (^{14}C)GABA (Redburn et al 1976, Redburn 1977). A similar release of radioactivity has also been found in rat and chick whole retinae preloaded with (^3H)GABA (Voaden and Starr 1972, Starr 1975, López-Colomé et al. 1978) as well as release from labelled horizontal cells in goldfish (Yazulla 1983). In two studies of the rabbit retina (Bauer and Ehinger 1977, Bauer 1978), a light-induced release of (^3H)GABA has been demonstrated, an important criterion for establishing a neurotransmitter role for GABA.

Effects of GABA

Acetylcholine, which is located in a subset of amacrine cells, is also a neurotransmitter candidate in the retina (Baughman and Bader 1977, Masland and Mills 1979, Neal 1984).

In the rabbit, GABA inhibits the release of acetylcholine (Massey and Neal 1979, Massey and Redburn 1982, Cunningham and Neal 1983) suggesting an interaction between GABAergic and cholinergic neurons. However, it has not been possible to demonstrate the site of action of GABA: whether the effect is mediated through a chain of neurons, or whether it occurs by means of GABA receptors located on the cholinergic neurons.

Finally, it has been shown that the GABA antagonists, bicuculline and picrotoxin, abolish the direction sensitivity of movement detecting ganglion cells (Wyatt and Daw 1976, Caldwell et al. 1978, Daw et al. 1982), suggesting that GABA neurotransmission participates in the movement detection system in the retina (Bonaventure et al. 1983).

Aim of the Study

There is evidence suggesting GABA as a neurotransmitter in the retina. However, this evidence is far from conclusive, and little is known about the neuronal circuitry in which GABA acts. Therefore, the aim of this work was to further characterize the neurotransmitter role of GABA in the retina, and to demonstrate the cells which use GABA as a neurotransmitter. The investigations performed to achieve this goal were:

- examinations of the uptake of GABA from a morphological point of view. Since GABA uptake occurs also in glial tissue, the specific purpose of this part was to investigate the usefulness of some GABA analogues in visualizing GABA neurons in mammals.

- studies on the mechanisms for the release of (^3H)GABA from the rabbit retina, and in addition, demonstrations of receptors for other neurotransmitters located presynaptically to and affecting GABA neurons.

- studies of the postsynaptic effects of GABA on cholinergic neurons in a species not previously investigated, the chick, and demonstrations of GABA receptors located on the cholinergic neurons.

MATERIALS AND METHODS

Animals.

Retinae from goldfish, chick, rat, guinea-pig, rabbit, and man were processed for autoradiography. Rabbit retinae were used in the uptake studies of (^3H)isoguvacine. (^3H)GABA release was studied in rabbit retinae, and the effects of GABA on (^3H)acetylcholine release in chick retinae.

Autoradiography

Intraocular injections. Light adapted or dark adapted eyes of different species were injected intravitreally with (^3H)GABA, (^3H)muscimol, (^3H)nipecotic acid, or (^3H)isoguvacine. After 30 min, 4 hours, or 24 hours, the animals were sacrificed, the eyes enucleated, the vitreous carefully removed, and the posterior segments cut into small pieces. The samples were freeze-dried, fixed in dry gaseous formaldehyde at 80°C for 1 hour, embedded in plastic, cut at 4 μm , and covered with autoradiographic stripping film. The procedure has been described previously and was demonstrated to be free from diffusion or extraction artefacts at the light microscopic

level (Ehinger and Falck 1971). After 1-12 weeks of exposure, the autoradiograms were developed and fixed.

Incubations. Pieces of the posterior segment were incubated in a balanced salt solution (Ames and Nesbett 1981). After 10 min, (^3H)isoguvacine, (^3H)muscimol, or (^3H)nipecotic acid was added and the incubations continued another 15, 30, 45, or 60 min.

In a second series of experiments, the retinae were preincubated in GABA, ouabain, or bicuculline before and during the exposure to (^3H)muscimol.

In a third, series they were preincubated in GABA, beta-alanine, taurine, or glycine before and during the exposure to (^3H)isoguvacine.

Uptake studies

Pigmented rabbits were sacrificed by an air injection into the ear vein. The retinae were gently peeled off from the choroid. (^3H)isoguvacine was added after 30 minutes of preincubation in the balanced salt solution (37°C or 0°C) with or without GABA, beta-alanine, taurine, glycine, or ouabain. After an additional 30 min (except for the time course study), the retinae were washed at 2 x 5 min at 0°C, weighed, and homogenized in distilled water.

To study uptake kinetics, various amounts of non-radioactive isoguvacine were added together with (^3H)isoguvacine.

Release studies

Eyes from pigmented rabbits were injected intravitreally with (^3H)GABA. After 30 min or 4 hours, the rabbits were sacrificed by an air embolus in the ear vein. The eyes were enucleated, the vitreous carefully removed, and the posterior segment everted and placed in a water jacketed superfusion chamber. The retinae were superfused with the balanced salt solution to which aspartate, glutamate, glycine, GABA, or some agonists were added for 2 min. The effect of K^+ was studied in the presence or absence of extracellular Ca^{++} , and the effect of ouabain was also analyzed.

Similarly, chick retinae were injected with (^3H)choline intravitreally. After 1 hour, the chicken were decapitated, and the eyes were enucleated and processed as described above. The effect of K^+ in the presence or absence of extracellular Ca^{++} as well as the effects of GABA, its antagonists, or agonists were studied. The application times were 2 min for K^+ and 15 min for the other substances.

Measurements of radioactivity

The radioactivity of the samples was measured by liquid scintillation spectrometry. Quench corrections were obtained with the external standard channels ratio method.

Statistical Methods

The statistical methods used were Student's two tailed t-test in uptake studies and Mann-Whitney's two tailed ranking test in release studies.

Electrophysiology

A coculture of staged embryonic chick retinal cells and postnatal rat muscle cells was used. Myoblasts were obtained from the hindlimbs of postnatal day 1 rats, dissociated and cultured in dishes, where they were allowed to fuse into myotubes. Dissociated retinal cells from chick embryos were harvested (0.05 to 10×10^6 cells per dish) and cocultured with the myotubes (fig. 1). In a couple of hours the retinal neurons form functional synapses with the muscle cells (Puro et al. 1977).

Intracellular recordings from the myotubes were obtained by KCl-filled micropipets (fig. 2) and GABA, isoguvacine, muscimol, bicuculline, or flurazepam were applied near the retinal neurons by microiontophoresis.

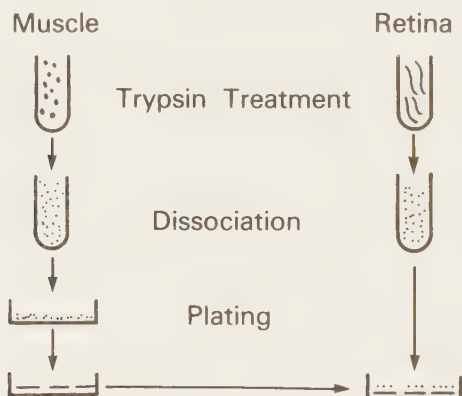


Fig. 1. Schematic outline for the preparation of retina-muscle cocultures. After five to seven days retinal cells from chick embryos were added to the muscle cultures.

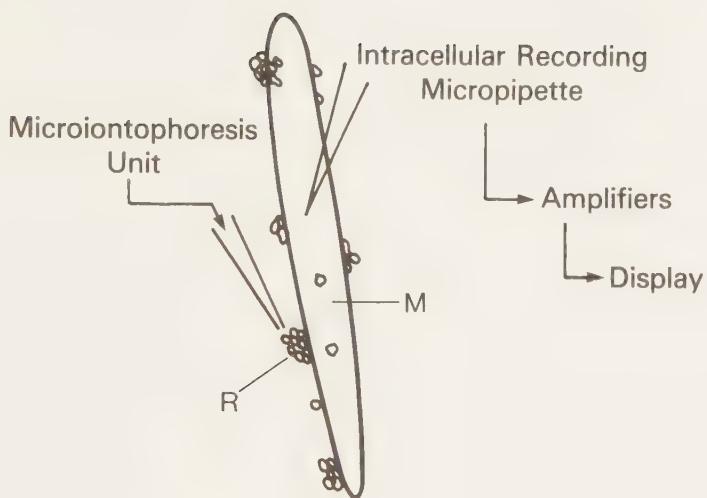


Fig. 2. Schematic drawing of the experimental set-up. Intracellular recordings from the muscle cells (M) were obtained with a KCl-filled micropipet and GABA was applied microiontophoretically. R = retinal cells.
From Yeh et al. (1983).

RESULTS AND COMMENTS

The usefulness of GABA analogues in morphological studies of GABA neurons

In autoradiographic studies of the retina, (^3H)GABA accumulates in glial cells as well as in neurons, particularly in the mammalian retina (Neal and Iversen 1972, Bruun and Ehinger 1974, Marshall and Voaden 1974a, 1975, Ehinger 1977). In order to avoid the obscuring glial labelling we studied the morphological uptake of some GABA-like substances (I). Two GABA agonists, muscimol and isoguvacine, and the GABA uptake inhibitor, nipecotic acid, were available in tritiated form. For comparison, some retinæ were exposed to (^3H)GABA as well.

(^3H)GABA. Four hours after an intravitreal injection of (^3H)GABA into rabbit eyes, the radioactivity was located in amacrine neurons, in the inner plexiform layer, and in some ganglion cells. The glial cells were only weakly labelled. These results agree with a previous study (Ehinger 1977) where, by prolonging the exposure time to (^3H)GABA, the initial heavy glial labelling gradually disappeared from the retina. In goldfish retinæ, the same structures were labelled, as were, in addition, horizontal cells. This is also in agreement with previous studies (Lam and Steinman 1971, Marc et al 1978).

(^3H)Muscimol. Rabbit, guinea-pig, and goldfish eyes were intravitreally injected with (^3H)muscimol. Pieces of the posterior segments from other eyes were also incubated in this radioligand. In rabbit and goldfish, the uptake pattern

was similar to that of (^3H)GABA. Cells corresponding in position to amacrine cells, the inner plexiform layer, and some ganglion cells were labelled. However, no clear labelling of horizontal cells could be demonstrated in goldfish retinæ. Most of the retinæ examined were attached to the pigment epithelium. In a study by Yazulla and Brecha (1980), horizontal cells were weakly labelled in goldfish retinæ dissected free from the pigment epithelium. One explanation for the unlabelled horizontal cells in the present study could be a poor penetration of (^3H)muscimol through the retina and the pigment epithelium barrier. Marshall and Voaden (1974b) reported an enhanced uptake of (^3H)GABA into horizontal cells of avian retinæ detached from the pigment epithelium. Similar observations were made by Pourcho et al. (1984) in the mudpuppy retina.

In guinea-pig retinæ, the labelled structures were similar to those of the rabbit retina. In both these mammals there was also a diffuse radioactivity throughout the retina, compatible with glial uptake.

The uptake pattern with labelled cell bodies indicated that (^3H)muscimol could not be characterized as a pure GABA receptor marker as was previously postulated in the brain (Chan-Palay 1978). To further investigate a possible uptake of (^3H)muscimol, the retinæ of rabbit and goldfish were preincubated in GABA, glycine, bicuculline, or ouabain. In addition, rabbit retinæ were incubated at 0°C . GABA significantly reduced the labelling, whereas bicuculline had a weak effect, and glycine almost none. In the presence of ouabain, a blocker of the Na^+ , K^+ -ATPase necessary for neurotransmitter uptake (Iversen 1975), or at 0°C , only diffuse background

radioactivity could be detected in the retinae. The results indicate that (^3H)muscimol is taken up into retinal cells in agreement with some recent studies (Yazulla and Brecha 1980, Pourcho 1981, Blanks and Roffler-Tarlov 1982, Pourcho and Goebel 1983). The results further suggest that the uptake process could be associated with the transport of GABA, but not with that of glycine. In mouse retinae, (^3H)muscimol did not accumulate in glial cells beyond the background level (Blanks and Roffler-Tarlov 1982), but in our study we found some glial labelling in the rabbit and guinea-pig retinae. Muscimol can thus be of some use in morphological studies of GABA neurons, but there seems to be no obvious advantage of (^3H)muscimol over (^3H)GABA.

(^3H)Nipecotic acid. Rabbit eyes were intravitreally injected with (^3H)nipecotic acid. Pieces of the posterior segments of other eyes were also incubated in this substance. Nipecotic acid is a GABA uptake inhibitor, with greater inhibition of glial than of neuronal uptake systems in mouse and rat brain slices (Schousboe et al. 1979). In our study, (^3H)nipecotic acid accumulated mainly in glial cells, and it is therefore not useful in studies of the present type of GABA neurons.

(^3H)Isoguvacine. In the primary study (I) of rabbit retinae, (^3H)isoguvacine was located in amacrine cells, in the inner plexiform layer, and in some ganglion cells. Almost no glial labelling was detected. Labelling with (^3H)isoguvacine was found particularly useful in experiments in vitro in which (^3H)GABA was previously shown to give mainly glial labelling (Ehinger 1977). The results suggest that (^3H)isoguvacine, previously known mainly as a GABA receptor agonist (Krogs-

gaard-Larsen and Johnston 1978, Krogsgaard-Larsen et al. 1980), could be useful for the specific purpose of demonstrating GABA neurons without too much obscuring glial uptake. The morphological study of (^3H)isoguvacine uptake was thus extended to include different species (II).

Goldfish, chick, rat, guinea-pig, rabbit, and human eyes were intravitreally injected with (^3H)isoguvacine. Pieces of the posterior segments of other eyes were also incubated in this substance. Labelling was found in cells with the position of amacrine neurons, in the inner plexiform layer (with the innermost part most heavily marked in all species except man), and in ganglion cells to a variable degree. In goldfish and chick retinæ, horizontal cells and the outer plexiform layer were labelled as well (fig. 3). No interfering glial labelling could be seen. In human, guinea-pig, and rat retinæ it has been very difficult to visualize presumed GABA neurons because of obscuring glial labelling (Neal and Iversen 1972, Bruun and Ehinger 1974, Marshall and Voaden 1974a). Thus, the neuronal labelling of (^3H)isoguvacine is in agreement with that of (^3H)GABA in those species where this can be studied, i.e. goldfish, chick, and rabbit. In addition, (^3H)isoguvacine visualizes neurons in species where the glial labelling of (^3H)GABA is too pronounced to allow neurons to be distinguished, i.e. rat, guinea-pig, and man. The study suggests that there are differences in glial and neuronal GABA uptake systems, and that isoguvacine is a useful marker for presumptive GABA neurons.

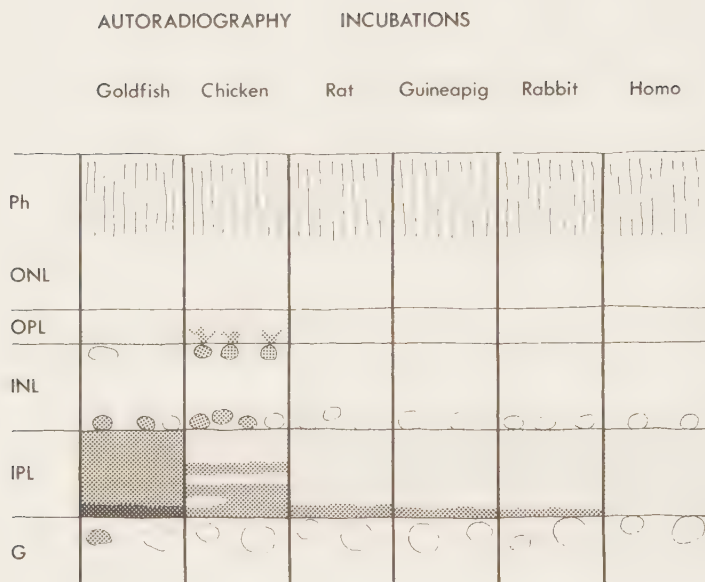


Fig. 3. Schematic drawing of the distribution of radioactivity in retinae incubated in (^3H)isoguvacine for 15 min. In all species, the radioactivity appeared in the inner plexiform layer, in amacrine cells, and in ganglion cells. In goldfish and chick, the horizontal cells and the outer plexiform layer were also labelled. Ph, Photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer.

The uptake of (³H)isoguvacine in the rabbit retina

The uptake of (³H)isoguvacine (3.1×10^{-9} M) was further explored in rabbit retinæ, using quantitative radiochemical techniques (III).

The tissue/medium uptake ratio was low, about 3, compared to that of GABA, for which it is about 12-18 in rabbit retina (Neal 1976, Agardh and Ehinger unpublished data) and at least 12 in rat retinæ (Starr and Voaden 1972, Goodchild and Neal 1973). Since rabbit retinæ incubated in (³H)isoguvacine accumulate radioactivity almost exclusively in neurons and in the inner plexiform layer, and since rabbit and rat retinæ incubated in (³H)GABA accumulate radioactivity in glial cells to a great extent (Marshall and Voaden 1975, Ehinger 1977), the high uptake ratio for (³H)GABA may reflect mainly glial accumulation.

The neurotransmitter uptake systems are energy-dependent (Iversen 1975), and hence the uptake of (³H)isoguvacine was studied at 0°C or in the presence of ouabain. The uptake of (³H)isoguvacine was significantly reduced ($p < 0.001$) both at 0°C and in the presence of ouabain. This indicates that (³H)isoguvacine is accumulated by an active, energy-dependent transport mechanism across the cell membrane.

To further investigate if isoguvacine uses the same transport system as GABA does, the retinæ were preincubated in GABA, beta-alanine, taurine, or glycine before the exposure to (³H)isoguvacine. Both GABA and beta-alanine (10^{-3} M) reduced the uptake significantly ($p < 0.001$). The effect of GABA was more pronounced, and a reduction of the isoguvacine uptake was also demonstrable with 10^{-4} M GABA ($p < 0.05$): in contrast, beta-alanine had no significant effect at 10^{-4} M. The action of beta-alanine can be explained by a structural

resemblance to GABA. Beta-alanine is not likely to be a true retinal neurotransmitter, but is accumulated in amacrine cells and in the inner plexiform layer in a manner similar to that of GABA (Bruun et al. 1974). Glycine and taurine did not affect the uptake of (^3H)isoguvacine. The results indicate that (^3H)isoguvacine is taken up by the GABA transport system, which is in agreement with a recent study on rat brain synaptosomes (White and Snodgrass 1983).

In the morphological experiments, (^3H)isoguvacine accumulated in neurons in a manner that indicated a highly specific uptake. Therefore, the uptake kinetics were studied further in test tube experiments, and the results were analyzed in Lineweaver-Burk plots. The uptake was found to be approximately linear between 5 and 40 min, and the uptake velocity was therefore measured in 30 min incubations, and with isoguvacine concentrations ranging from 10^{-9} - $4 \times 10^{-5}\text{M}$. Over this concentration range, no clear breakpoint between high and low affinity uptake systems was evident in the Lineweaver-Burk plot. The uptake system did not seem to be easily saturated, even at higher concentrations (unpublished data). Thus, there seems to be considerable overlaps between high and low affinity uptake systems. Nevertheless, K_m values for the concentration range from 10^{-9} - $4 \times 10^{-5}\text{M}$ were calculated. If the uptake was regarded as a single, simple process a K_m value of $1.93 \times 10^{-7}\text{M}$ was obtained, but if the plot was arbitrarily split into two segments, 10^{-9} - 10^{-7}M and 10^{-6} - $4 \times 10^{-5}\text{M}$, K_m values of $6.5 \times 10^{-8}\text{M}$ and $5.8 \times 10^{-5}\text{M}$ were obtained.

The uptake studies of (^3H)isoguvacine thus indicate that it is taken up with an energy-dependent transport system intended for GABA. Most likely, at low concentrations, there

is a high affinity uptake, although no clear distinction between high and low affinity uptake systems could be demonstrated. The difference in uptake ratios between (^3H)isoguvacine and (^3H)GABA may reflect differences in the amount of uptake into glial cells.

(^3H)GABA release

The release of (^3H)GABA was studied in rabbit retinae (IV). Since (^3H)GABA is localized mainly in glial cells at half an hour after an intravitreal injection and mainly in neurons at 4 hours after the injection (Ehinger 1977), it was possible to compare glial and neuronal release of (^3H)GABA.

The effect of K^+ . 40mM K^+ caused an increased release of radioactivity from retinae in which mainly neurons were radioactive ($p < 0.01$), but not from retinae where mainly glial cells were labelled. This is in agreement with a study of the rat retina by Neal and Bowery (1979). After blockade of non-specific GABA uptake, Sarthy (1983) reported a small release of radioactivity from retinae incubated in (^3H)GABA, and suggested the release to be of glial origin. However, the evidence for this origin was indirect and a neuronal source was not excluded.

Calcium plays an important role in neurotransmitter release by exocytosis (for a review, see Kelly et al. 1979). The effect of 40mM K^+ was therefore studied in the absence of Ca^{++} , and EGTA was also added in order to bind tissue Ca^{++} . The release was decreased, but the change was not statistically significant. Thus, 40mM K^+ was able to release radioactivity also in the absence of Ca^{++} . Further, blockade of the Ca^{++} -mediated release with Mg^{++} , did not inhibit the effect

of K^+ . Thus, there seems to be a Ca^{++} -independent release of (3H)GABA from amacrine cells. A similar release has also been demonstrated in rabbit retinal homogenate fractions (Redburn et al. 1976), and comparable results were obtained in intact rat retinae (Voaden and Starr 1972), although in these cases it was not clear that the amacrine cells were the source of the radioactivity released.

A Ca^{++} -independent release of (3H)GABA has been demonstrated in retinal cells with very few or no synaptic vesicles, the horizontal cells of toad (Schwartz 1982) and goldfish (Yazulla and Kleinschmidt 1983). This suggests a release from a non-vesicular cytoplasmic neurotransmitter pool. In the present study of the rabbit retina (IV), the Ca^{++} -independent release emanated from amacrine cells, and it should be noted that these cells contain numerous vesicles. This may indicate that both vesicular and non-vesicular release systems can be present in one and the same cell.

The effects of amino acids. The interactions between retinal neurons with different transmitters is not very well known. The amacrine cells are connected with bipolar cells and ganglion cells, but, in addition, they have numerous contacts with each other (for a recent review, see Dowling and Dubin 1984). Glutamate and aspartate have been proposed as transmitters in the plexiform layers (Redburn 1981, Altschuler et al. 1982, Miller et al. 1982, Brandon and Lam 1983). Therefore, the effects of glutamate, aspartate, and a number of their respective receptor agonists were studied. None of them affected the release of (3H)GABA from the retina. Most likely, therefore, there are in the rabbit retina no glutamate or aspartate receptors on the GABA-accumulating neurons or on

neurons connected to these cells.

Different results have been obtained in other species, which may suggest species differences. In rat (Kamada et al. 1981) and chick (Morán and Pasantes-Morales 1983), glutamate was found to increase the release of radioactivity from retinæ preloaded with (^3H)GABA. However, rat retinal glial cells accumulate (^3H)GABA to a great extent, and the release could thus emanate from glia. In chick retina, the glutamate concentration used has been demonstrated to cause spreading depression (van Harreveld and Fifkova 1971, van Harreveld 1977, 1978) making it difficult to interpret the results. Thus, the evidence for species differences is suggestive but not conclusive.

The effects of the inhibitory amino acids, GABA and glycine, were investigated as well. Both are neurotransmitter candidates in amacrine cells (for recent reviews, see Ehinger 1982, Brecha 1983), and thus it was of interest to study a possible interaction between cells with the same neurotransmitter, GABA, or between cells with different neurotransmitters, GABA and glycine. Therefore, in a first set of experiments we examined how GABA, some GABA analogues, or glycine influenced the spontaneous neuronal release of radioactivity. No inhibition of the resting release could be demonstrated by GABA, by its analogues or by glycine. GABA caused an increased release of radioactivity instead, which was interpreted to be due, most likely, to a homoexchange.

In a second set of experiments, we studied how GABA and its analogues affected the K^+ -induced release. With the exception of isoguvacine, none of them influenced the retinal response to K^+ . Thus, it was not possible in this study (IV)

to demonstrate with certainty any GABA or glycine receptors presynaptic to GABA accumulating neurons.

Postsynaptic effects of GABA

In rabbit retinae, the postsynaptic effects of GABA on the acetylcholine release have previously been extensively studied. An inhibitory effect of both the spontaneous and the light-induced release has been demonstrated (Massey and Neal 1979, Neal and Massey 1980, Massey and Redburn 1982, Cunningham and Neal 1983; for a recent review see Neal 1984). It was therefore of interest to study a possible interaction between these transmitters in a different species, the chick.

GABA-mediated inhibition of (³H)acetylcholine release from intact retina. In the present study (V), the spontaneous release of (³H)acetylcholine could not be directly inhibited by GABA. However, by blocking GABA receptors with bicuculline, or Cl⁻channels with picrotoxin, the spontaneous release increased. This indicates that the cholinergic neurons are tonically inhibited by a continuous release of endogenous GABA.

Further, the GABA effect on a stimulus-induced (³H)acetylcholine release was explored. Light flashes did not influence the release of (³H)acetylcholine from the chick retina in vitro as was previously observed in the rabbit retina in vivo (Massey and Neal 1979, Neal and Massey 1980, Massey and Redburn 1982, Cunningham and Neal 1983). The explanation could be a species difference, but, like in our experiments, no light-induced release was found in the rabbit retina in vitro (Neal, personal communication). Thus, the most plausible explanation is the use of differing experimental proce-

dures.

An increase of the K^+ -concentration in the superfusing medium from 3.6 to 8.6M caused an increased release of radioactivity from the retina ($p < 0.01$). This K^+ -concentration is not high enough to cause the spreading depression which can occur in the chick retina (van Harreveld 1977).

The GABA effects on the K^+ -induced release of radioactivity were therefore further investigated. Neither GABA, nor its agonists, isoguvacine or muscimol, inhibited the K^+ -induced release. When the GABA receptors were blocked with bicuculline before stimulation with K^+ , there was no further increase of the K^+ -induced release beyond the background level caused by bicuculline. This would have been expected, if the K^+ -induced release were controlled by GABA. The results thus indicate that cholinergic neurons are tonically inhibited by GABA, but that GABA does not control the stimulus-induced release caused by K^+ .

GABA-mediated inhibition of acetylcholine release from neurons in culture. The ability of embryonic chick retinal neurons to form transient synapses with a postsynaptic target, a muscle cell in culture, was used in this study (VI) (Puro et al. 1977). The spontaneous firing rate of the muscle cell was an indication of the response to acetylcholine released from cholinergic neurons (Puro et al. 1977). Both GABA and its agonists, isoguvacine and muscimol, inhibited the firing rate of the muscle cell when applied by microiontophoresis close to the innervating retinal neurons. Since bicuculline reversibly blocked this inhibition, GABA most likely mediates its effects on synaptic transmission via GABA receptors. With

this technique, the inhibitory effect of GABA on one single neuron could be detected.

Benzodiazepines, like flurazepam, are known to facilitate GABAergic transmission (for a short recent review, see Richards and Möhler 1984). A potentiation of the inhibitory effect of GABA was demonstrated in the present experiment. This finding is the first direct evidence that benzodiazepine-GABA interactions can influence information processing by retinal neurons.

It thus appears that GABA inhibits the release of acetylcholine from chick retinal neurons by acting via receptors located directly on the cholinergic neurons.

CONCLUDING REMARKS

In the retina, exogenously applied GABA accumulates not only in neurons, but also in glial cells. In studies of the GABA neurotransmitter system, a specific marker for neurons would be of great value. Three GABA mimicking substances, muscimol, isoguvacine, and nipecotic acid were investigated with autoradiographical techniques (I), and isoguvacine was shown to be useful for the specific purpose of labelling presumed GABA neurons. In all species examined (II), isoguvacine demonstrated a morphological uptake pattern similar to that of GABA. Visualization of presumed GABA neurons could thus be demonstrated in rat, guinea-pig, and man. In these species, no clear identification of GABA neurons had been described previously.

The quantitative uptake of isoguvacine was further studied (III) to assess its usefulness as a marker for GABA systems. The results indicate an energy-dependent uptake by means of the GABA transport process, and most likely, the existence of a high affinity uptake system.

Lately, two separate mechanisms for the release of neurotransmitters have been proposed, one which is Ca^{++} -dependent and one which is not. The Ca^{++} -dependent release is thought to originate from synaptic vesicles and the Ca^{++} -independent one from a non-vesicular pool. In this study of the retina (IV), a Ca^{++} -independent, K^{+} -induced release of (^3H)GABA was demonstrated, originating from amacrine cells which contain synaptic vesicles. The mechanism for and sub-cellular origin of this release remains to be established.

Little is known about the interaction between retinal neurotransmitters. In this study of (^3H)GABA release from the rabbit retina (IV), the effects of some other putative neurotransmitters were investigated. The substances tested were glutamate, aspartate, glycine, and GABA itself. The results suggest that there are no corresponding receptors located presynaptically to the (^3H)GABA accumulating neurons.

Benzodiazepines are known to influence GABA systems in the brain, but this has not been demonstrated previously in the retina. The present results (VI) suggest that these drugs may affect vision already at the retinal level.

An interaction was demonstrated between cells using GABA and cells using acetylcholine as their presumed neurotransmitters (V, VI). The cholinergic neurons were tonically inhibited by a continuous release of endogenous GABA, and the inhibition occurred at receptors located on the cholinergic neurons. The demonstration of a continuous neurotransmitter release suggests that the now classical theory of stimulus-induced quantal release of neurotransmitters in millisecond bursts should be supplemented with one including also a continuous background release of at least some of the neurotransmitters. This is of importance for furthering our understanding of regulatory mechanisms not only in the retina, but in all nervous tissue.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to:

Professor Berndt Ehinger, my adviser, for eminent guidance and generous and enthusiastic support during the course of this study.

Dr Donald Puro for an excellent introduction into, and expert guidance within the field of electrophysiology.

Docent Birgitta Bauer for prolific collaboration.

Dr Hermes Yeh and Dr Roland Herrmann for fruitful collaboration.

Mrs Anitha Bruun, Miss Elisabet Birath, Miss Annett Forsberg, and Miss Gunilla Jakobsson for skilful technical assistance.

Mrs Maj-Britt Andersson for excellent secretarial help.

Miss Louise Goldberg for skilfully preparing photographs and illustrations.

This study was supported by grants from:

The Swedish Medical Research Council (project 14X-2321)

The Faculty of Medicine, University of Lund

The Magnus Bergwalls Stiftelse

The Carmen and Bertil Regnérs Fond

The Swedish Society of Medical Sciences

The Helfrid and Lorentz Nilssons Stiftelse

The Thorsten and Elsa Segerfalks Stiftelse

The Anna Lisa and Sven-Eric Lundgrens Stiftelse

The Torsten and Ragnar Söderbergs Stiftelse

The Herman Järnhardts Stiftelse

The Ebba Henrikssons Fond

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(³H)-Muscimol, (³H)-Nipecotic Acid and (³H)-Isoguvacine as Autoradiographic Markers for GABA Neurotransmission

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With 8 Figures

Received October 16, 1981

Summary

Retinas from rabbit, goldfish and guinea-pig were exposed to (³H) GABA, (³H)-muscimol, (³H)-nipecotic acid and (³H)-isoguvacine either by intravitreal injection *in vivo* or by incubations in a balanced salt solution and the distribution of radioactivity was then studied with autoradiography. All substances labelled a similar set of presumed amacrine cells. Incubating at 0 °C, in 10⁻⁵ M ouabain, or in 10⁻³ M GABA inhibited the labelling by (³H)-muscimol whereas bicuculline (10⁻⁴ M), and glycine (10⁻³ M) were less efficient blockers. The result is interpreted as a neuronal uptake of (³H)-muscimol rather than as a GABA receptor binding. All the substances except (³H)-isoguvacine also labelled glia to such a degree that neuronal labelling was often disguised in rabbits and goldfish. Glial labelling by muscimol was less pronounced in guinea-pig. (³H)-isoguvacine (tested only in rabbits) gave a strong labelling of cells with the distribution of GABA neurons and only little glial labelling.

Introduction

(³H)-GABA has been shown to be accumulated in rabbit retina in a set of amacrine cells which seem likely to use GABA as their neurotransmitter (Ehinger and Falck, 1971; Iversen and Schon, 1973; Brandon, Lam, and Wu, 1979). However, there is also a considerable uptake of (³H)-GABA in glia so that with short exposure times, the neurons

cannot readily be discerned (Neal and Iversen, 1972; Ehinger, 1977) and this is a problem not only in the retina but also in the brain (Hökfelt and Ljungdahl, 1972; Iversen and Kelly, 1975; Höslí and Höslí, 1978). In rabbits and a few more species the glial radioactivity disappears faster than the neuronal so that the neurons will eventually stand out (Ehinger, 1977), but in many mammalian species (including rats and guinea-pigs) this is not the case. (^3H)-GABA is therefore not universally useful as a marker for GABA neurons.

Both receptor binding and neuronal uptake of labelled transmitter are conceivable as a basis for autoradiographic neuron labelling. The GABA agonist, (^3H)-muscimol, was introduced as a GABA receptor ligand suitable for autoradiographic demonstration (Chan-Palay, 1978). However, as will be shown, it more likely demonstrates neuronal uptake than receptor binding in our test system. Two other substances seem of interest as possible GABA neurons markers: the uptake inhibitor, nipecotic acid, and the GABA agonist, isoguvacine. The work reported here shows that neither the former nor muscimol gives any distinct labelling in rabbit or goldfish, but isoguvacine does. A few pilot experiments have also been done in rabbits with the GABA uptake inhibitor, (^3H)-ACHC (kindly donated by N. G. Bower), but only diffuse distribution of the radioactivity was seen. This is in contrast with frog retina, where the drug is accumulated by GABA neurons (Neal, Cunningham, and Marshall, 1979).

Materials and Methods

Light adapted rabbit eyes were injected intravitreally with 50 μCi of (^3H)-GABA, 50 μCi (^3H)-muscimol, 20 μCi (^3H)-nipecotic acid or 20 μCi (^3H)-isoguvacine. After four hours, small pieces of the posterior eye segments were freeze-dried at -35°C in a tissue freeze-drier, fixed in gaseous formaldehyde at 80°C for one hour, embedded in plastic (Durcupan[®]), cut at 4 μ , and covered with autoradiographic stripping film. The procedure has been described previously (Ehinger and Falck, 1971) and was shown to be free from diffusion or extraction artefacts on the light microscopical level. After 1–12 weeks of exposure the autoradiograms were developed and fixed. The sections were counterstained with 1% toluidine blue in 40% ethanol as required.

Light adapted guinea-pig eyes were injected with 5 or 25 μCi (^3H)-muscimol or incubated in it (1.3 $\mu\text{Ci}/\text{ml}$) for 15 min at 37°C and also with 10^{-5} M ouabain. Tissue pieces were taken and processed as with rabbits.

Light adapted goldfish eyes (from 10–15 cm animals) were similarly injected with 5 μCi (^3H)-muscimol or 2, 5 or 10 μCi (^3H)-GABA and processed as above. 2, 5 or 10 μCi (^3H)-GABA was also injected into goldfish eyes, dark adapted for 16 hours. Injections and dissections were in these cases performed under scotopic conditions in dim red light.

In another set of experiments, small pieces of rabbit posterior eye segments were incubated at 37°C in 4 ml of a balanced salt solution (Ames, Parks, and Nesbett, 1980). After 10 min (^3H)-muscimol ($1.3 \mu\text{Ci/ml} = 1 \times 10^{-6} \text{ M}$), (^3H)-GABA ($1.3 \mu\text{Ci/ml} = 2 \times 10^{-7} \text{ M}$), or (^3H)-isoguvacine ($1.3 \mu\text{Ci/ml} = 3 \times 10^{-7} \text{ M}$) was added. The figures given are the final concentrations in the incubating solution. The incubations were continued for 15, 30, 45 or 60 min whereupon the tissue pieces were washed $2 \times 5 \text{ min}$ with the balanced salt solution at 0°C, without radioactive additives. Retinas were similarly incubated for 15 min in (^3H)-nipecotic acid, $2.5 \mu\text{Ci/ml} = 3 \times 10^{-7} \text{ M}$ (final concentration). The tissue pieces were then processed for autoradiography as described above. GABA (10^{-3} M), ouabain (10^{-5} M or 10^{-6} M), or bicuculline (10^{-4} M) were added to the salt solutions throughout the incubation in a further series of experiments with (^3H)-muscimol and rabbit retina. Glycine (10^{-3} M) was also tested, only in 10 min incubations but both at 37°C and 20°C.

Three pieces from at least four eyes were processed for each animal species, substance and dose when injected intravitreally. Incubations were performed with three tissue pieces from each of at least two (usually four) eyes for each species, drug and concentration.

4-amino-*n*-(2,3- ^3H)-butyric acid (GABA), 57 Ci/mmol and (*methylamine*- ^3H)-muscimol, 9.5 Ci/mmol were obtained from The Radiochemical Centre, Amersham. (^3H)-isoguvacine hydrochloride, 40.0 Ci/mmol and (ring- ^3H)-nipecotic acid, 46.0 Ci/mmol were obtained from New England Nuclear, Dreieichenhain, West Germany.

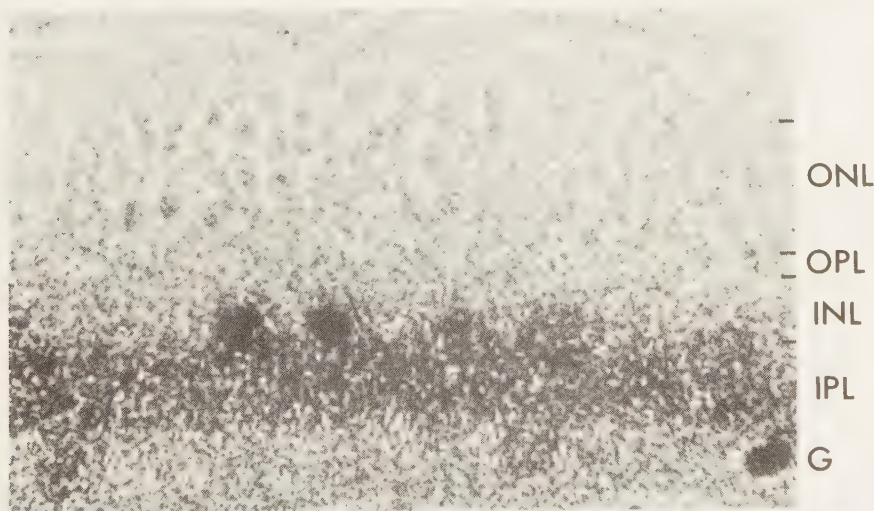


Fig. 1. Rabbit retina, $80 \mu\text{Ci}$ (^3H)-GABA intravitreally 4 hours. Radioactivity appears in the inner plexiform layer (IPL) in some cells with the position of amacrine cells. There is strong radioactivity in one ganglion cell and weaker in three others. ONL outer nuclear layer; OPL outer plexiform layer; INL inner nuclear layer; IPL inner plexiform layer; G ganglion cell layer. Phase contrast micrograph $\times 575$

Results

(^3H)-GABA, *intraocular injections, rabbits*. Four hours after the injection, radioactivity appeared mainly in the inner plexiform layer, in some cell bodies with the position of amacrine cells, and to a variable degree in ganglion cells (Fig. 1). The appearance has been described previously (Ehinger and Falck, 1971; Iversen and Schon, 1973; Brandon, Lam, and Wu, 1979) and the result is included here for comparison only.

(^3H)-GABA, *intraocular injections, goldfish*. Radioactivity appeared in the inner plexiform layer, often with some increase in the innermost parts and also often accumulated in "hot spots" suggesting highly radioactive small substructures within the layer. Radioactive cell bodies appeared among the innermost two or three cell rows of the inner nuclear layer, which is the position of amacrine cells. A few of these cells were quite large with stout processes reaching into the innermost part of the inner plexiform layer (Fig. 2). This is clearly the red-depolarizing, sustained type ("Ab amacrine cell") described by Murakami and Shimoda (1977), by Famiglietti, Kaneko, and Tachibana (1977) and by Lam and collaborators (Marc, Stell, Bok, and Lam, 1978; see also Lam *et al.*, 1980). No radioactivity was seen in the outer plexiform layer, either in dark- or light-adapted retinas. There was also



Fig. 2. Goldfish retina, $10\mu\text{Ci}$ (^3H)-GABA intravitreally 4 hours. Radioactivity appears in the inner plexiform layer and in cell bodies with the position of amacrine cells. One of them shows a stout process (arrow) reaching the innermost part of the inner plexiform layer. The horizontal cells are only weakly labelled. Designation of layers as in Fig. 1. Phase contrast micrograph $\times 200$



Fig. 3. Goldfish retina, 10 μ Ci (3 H)-GABA intravitreally 4 hours. Radioactivity appears in the inner plexiform layer, in some cell bodies in the inner nuclear layer and in some cells in the horizontal cell layer in the left half of the picture (arrows) but not in the right half. Designation of layers as in Fig. 1. Toluidine blue staining $\times 95$

some general radioactivity in the retina, the highest being in the innermost part and the lowest in the outermost parts.

With higher doses (10 μ Ci), the structures described above appeared, only more radioactive. In addition, some cells in the horizontal cell layer appeared (Fig. 3). These cells were much less radioactive than the cells at and in the inner plexiform layer. There were clear regional differences in the retina, central parts of it having radioactivity at the outer plexiform layer whereas peripheral parts showed less radioactivity in this location. This phenomenon was seen in both light- and dark-adapted retinas.

(3 H)-*Muscimol*, *intraocular injections, rabbits*. Four hours after the intraocular injection of (3 H)-muscimol (Fig. 4), there appeared an increase of radioactivity in the inner plexiform layer. Some cells with the position of amacrine neurons in the innermost part of the inner nuclear layer also became radioactive. They were not counted precisely, but appeared to constitute some 10 to 15% of the total number of cells in the innermost cells row. There was no obvious sublayering in the inner nuclear layer. Diffuse radioactivity was also apparent throughout the retina, more marked in the innermost part and less in the outer parts so that there was a gradient through the tissue, diminishing outwards. Ganglion cells were not seen to be more radioactive than their surround. There were no radial strands of radioactivity such as is at times seen when predominantly glia becomes labelled, but frequently cell bodies in the middle part of the inner nuclear layer were radioactive, suggesting that glial cells may have accumulated some radioactivity. There was no special radioactivity in the outer plexiform layer.

(3 H)-*Muscimol*, *incubations, rabbit*. These retinas in general

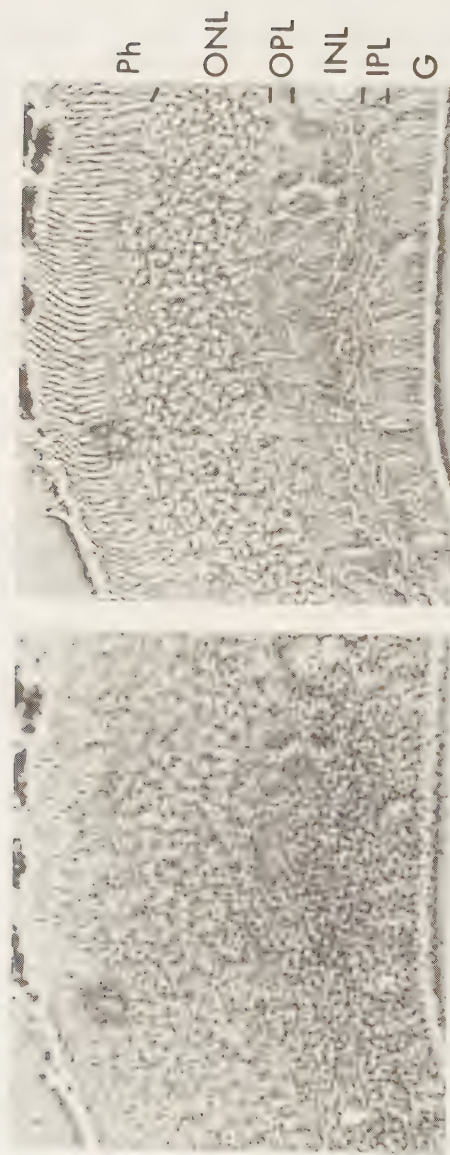


Fig. 4. Rabbit retina, $10\mu\text{Ci } (^3\text{H})\text{-muscimol}$ intravitreally 4 hours. Diffuse radioactivity is apparent throughout the retina with an increase in the inner plexiform layer and in some cell bodies with the position of amacines. Designation of layers as in Fig. 1. Left, focus on the silver grains; right, focus on the section. Phase contrast micrograph $\times 475$

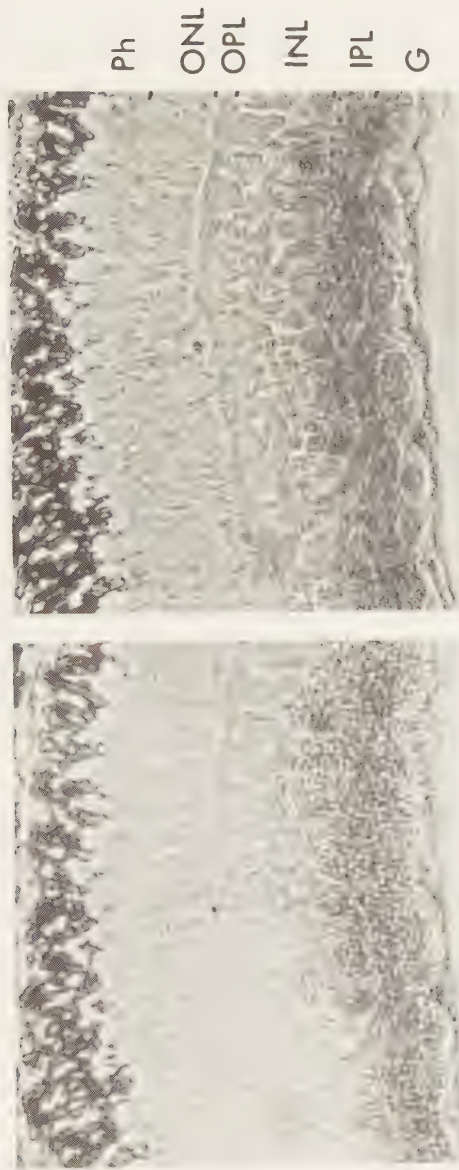


Fig. 5. Goldfish retina, $5 \mu\text{Ci}$ (^3H) -muscimol intravitreally 30 min. Diffuse radioactivity is seen throughout the retina with an increase in the inner plexiform layer and in some cells among the amacrine neurons. Designation of layers as in Fig. 1. Left, focus on the silver grains; right, focus in the section. Phase contrast micrograph $\times 475$

showed less radioactivity than after the intraocular injections, and the gradient from the vitreous surface towards the outermost layers was less marked. However, the inner plexiform layer still showed significant radioactivity, as did an unchanged number of cells among the amacrine neurons. All radioactive structures were the same as after the intraocular injections. Preincubating the retinas with ouabain or GABA (10^{-3} M) completely blocked the uptake of radioactivity in the inner plexiform layer and the cells in the inner nuclear layer. Bicuculline (10^{-4} M) had a moderate depressant effect on the accumulation of radioactivity in the inner plexiform layer and in the cells among the amacrine neurons. Glycine (10^{-3} M) had a variable effect, depressing the uptake weakly in some experiments but not in others.

(^3H)-*Muscimol*, *intraocular injections, goldfish*. The goldfish retinas displayed a distribution of radioactivity (Fig. 5) that was very similar to that in rabbits, *i.e.* it was seen mainly as a general radioactivity with a gradient decreasing from the vitreous surface towards the pigment epithelium and with an extra accumulation in the inner plexiform layer and in some cells among the amacrine neurons. Like in rabbits, there was no apparent sublayering in the inner plexiform layer, nor was there any special radioactivity in the outer plexiform layer.

(^3H)-*Muscimol*, *incubations, goldfish*. In these retinas, the differences in radioactivity between the outer and inner parts were much less pronounced than after intraocular injections, particularly when the retina had detached. The appearance of special radioactivity in the inner plexiform layer and in cells among the amacrine neurons was also much less evident. Pretreating the retina with ouabain (10^{-5} M) completely blocked the neuronal uptake of radioactivity whereas GABA (10^{-3} M) had some blocking effect but bicuculline (10^{-4} M) only weak and variable and glycine (10^{-3} M) none.

(^3H)-*Muscimol*, *intraocular injections and incubations, guinea-pig*. After the intraocular injections, radioactivity was seen in the inner plexiform layer and in cells with the position of amacrine cells and ganglion cells (Fig. 6), much like in rabbits. The inner third of the inner plexiform layer was at times more labelled than the outer two thirds. Both intensely and less intensely labelled cell bodies were seen among the amacrine cells and the ganglion cells. The glial uptake was considerably less than in rabbits or goldfish, making the neurons much more readily distinguishable. In the incubation experiments, the radioactivity was seen in the same structures as after intraocular injections. Ouabain (10^{-5} M) completely inhibited the uptake of radioactivity in the inner plexiform layer and in amacrine cells in incubation experiments.

(^3H)-*Nippecotic acid*, intraocular injections, rabbit. In these experiments there was a diffuse distribution of radioactivity with a gradient decreasing from the vitreal side towards the scleral. Radially directed strands, typical for Müller cells (glia) were common in specimens with high radioactivity (Fig. 7) as were accumulations of radioactivity at the level of the outer limiting membrane and in the middle of the

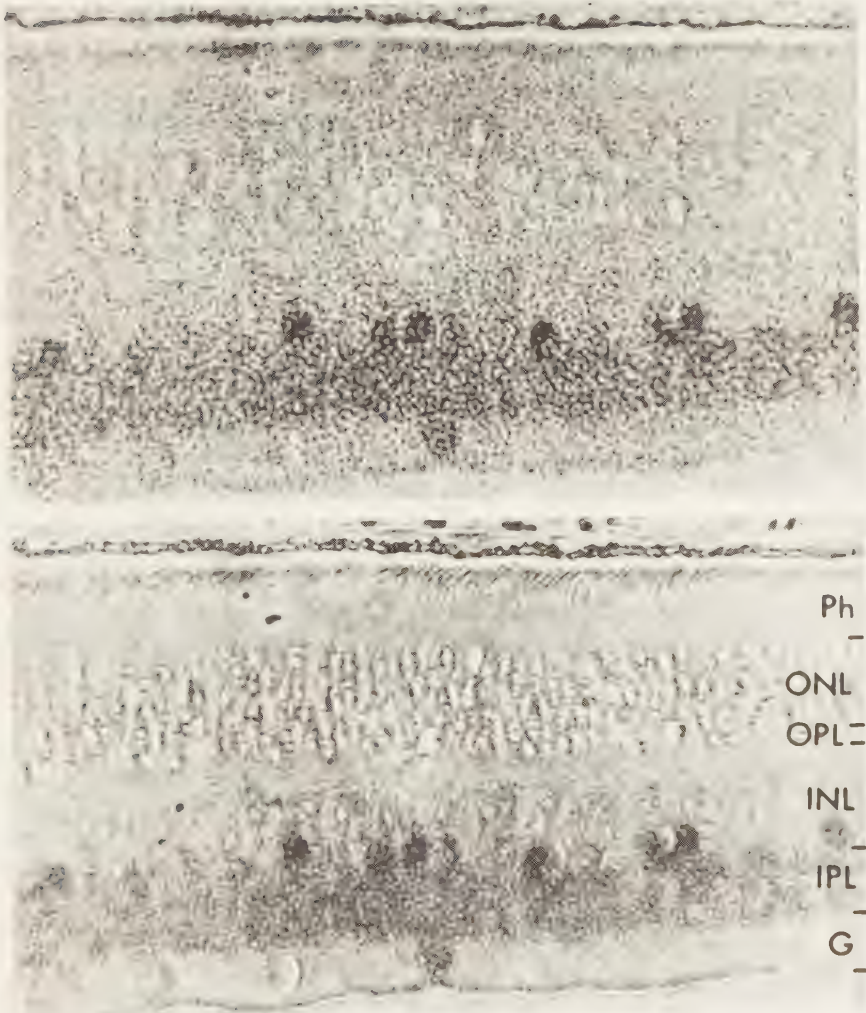


Fig. 6. Guinea-pig retina, $25\mu\text{Ci}$ (^3H)-muscimol intravitreally 4 hours. Radioactivity appears in the inner plexiform layer and in cell bodies with the position of amacrine and in ganglion cells. Designation of layers as in Fig. 1. Above focus on the silver grains; below focus on the section. Phase contrast micrograph $\times 475$

inner nuclear layer where the Müller cells have their cell bodies. Ganglion cells were as radioactive as their surround or slightly less. Most cell bodies in the innermost one or two cell rows of the inner nuclear layer showed some radioactivity. At times some cells (perhaps one or two per cent of the total number of cells in the innermost cell row of the inner nuclear layer) in this region were significantly more radioactive than the surrounding amacrine cells. There was usually no special accumulation of radioactivity in either of the plexiform layers although in specimens with high radioactivity some increase could be discerned in the inner plexiform layer.

(^3H)-Nipecotic acid, incubations, rabbit. With the incubation times used, mainly glial, diffuse labelling was apparent. Nerve cell bodies were only little or not at all more radioactive than the general background and always located as with the intraocular injections.

(^3H)-Isoguvacine, intraocular injections and incubations, rabbit. In these retinas, strong radioactivity appeared in the inner plexiform layer (Fig. 8) in three often poorly demarcated sublayers. The inner-

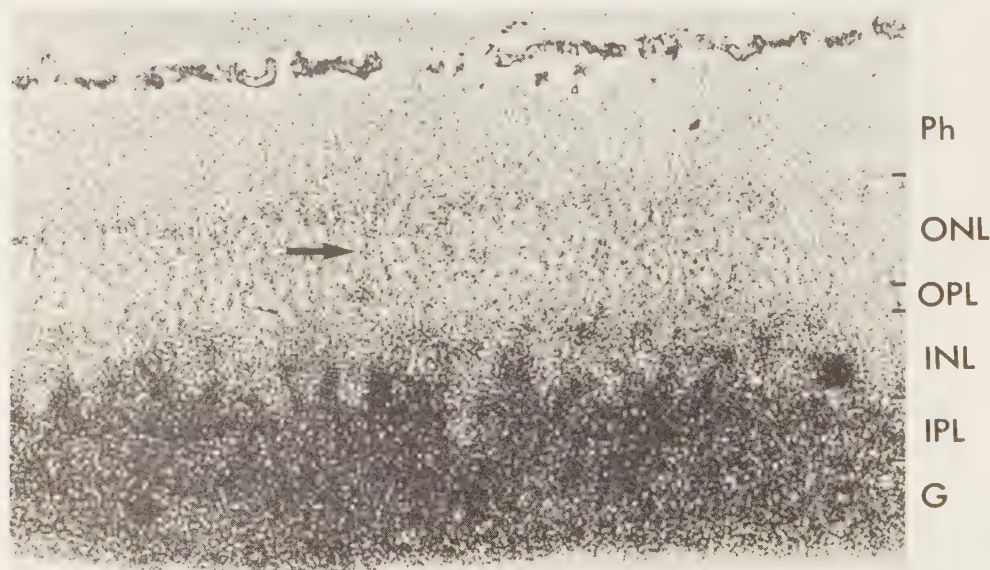


Fig. 7. Rabbit retina, $20\ \mu\text{Ci}$ (^3H)-nipecotic acid intravitreally 4 hours. Radioactivity appears in the inner parts of the retina and in a narrow layer at the outer limiting membrane demarcating the border between the photoreceptor layer and the outer nuclear layer. There is a tendency for the radioactivity to be accumulated in vertical strands (arrow). This is characteristic of the Müller cell radioactivity. The most strongly labelled cells could be neurons embedded in the radioactivity of the glia.

Designation of layers as in Fig. 1. Phase contrast micrograph $\times 450$

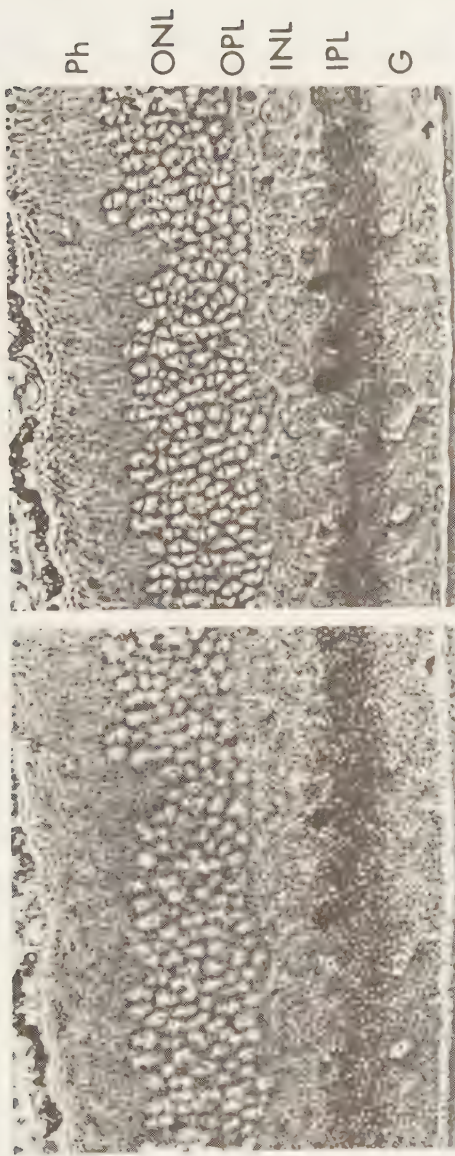


Fig. 8. Rabbit retina, 20 μ Ci (H)-isoguvacine intravitreally 4 hours. Sharply demarcated radioactivity appears in the inner plexiform layer and in some cells with the position of amacrine. There is comparatively little radioactivity in the glia. Designation of layers as in Fig. 1. Left, focus on the silver grains; right, focus on the section. Phase contrast micrograph $\times 475$

most was usually the most pronounced and the middle the least. The radioactivity was often accumulated in "hot spots" suggestive of large synaptic complexes. Radioactive cell bodies were also common in the inner part of the inner nuclear layer, *i.e.* among the amacrine neurons. About 20–30 % of the cells in the innermost cells rows were labelled. However, this figure is very hard to estimate more precisely because the labelled cells appear not only in the innermost cell row, but also further outwards. Strong labelling was seen in 30 % (127 out of 391 in the central retina) of the cells in the ganglion cell layer. These cells were usually of small to moderate size ($< 8 \mu$). Only weak labelling occurred in the other ganglion cells. Radioactive cells were also seen infrequently in the middle of the inner plexiform layer. They were of approximately the same size as the cells in the inner nuclear layer, or slightly less. Glia was only insignificantly labelled. There was no labelling in the layers outwards of the inner nuclear layer. The incubations showed the same picture as the intraocular injections, only with less radioactive labelling.

Discussion

Muscimol is a GABA agonist with high affinity for the GABA membrane receptor, about one magnitude higher than GABA itself (see, *e.g.*, Krogsgaard-Larsen and Johnston, 1978). It was initially felt that it might be useful for labelling postsynaptic GABA sites as was suggested by the report of only minimal affinity for the GABA uptake system (Johnston, 1971) and morphological observations (Chan-Palay, 1978; Chan-Palay and Palay, 1978). However, not only does (^3H)-muscimol label processes in the inner plexiform layer (where GABA-ergic synapses are presumed to occur in both rabbit and goldfish, see reviews by, *e.g.*, Lam *et al.*, 1980, or Ehinger, 1982) but also cell bodies among the amacrine cells. Synapses are rare on cell bodies which were therefore not expected to show up. Further, the distribution of the radioactive cell bodies (Fig. 4) was similar to that of the GABA-accumulating neurons (Fig. 1). It seems that the (^3H)-muscimol had been taken up into certain cells rather than labelled receptors. This suggestion is supported by the absence of radioactivity in retinas incubated at 0°C or with ouabain (which inhibits the $\text{Na}^+\text{-K}^+$ activated ATPase, depriving most membrane uptake mechanisms of their energy source). It is also supported by the observation that bicuculline (a potent GABA receptor blocker) was rather inefficient in inhibiting the cell labelling by (^3H)-muscimol, about as efficient as GABA. This effect of bicuculline may in fact be related to its inhibition of synaptosomal uptake of GABA which has recently

been reported (De Feudis and Somoza, 1977; De Feudis, Maitre, Ossola, Elkouby, Roussel, and Mandel, 1979) rather than to receptor blockade because, as discussed below, our procedure appears incapable of demonstrating GABA receptor binding. We therefore conclude that both *in vivo* and in incubations in a balanced salt solution, (^3H)-muscimol (or a metabolite, see Ott *et al.*, 1975, Snodgrass, 1978, Baraldi, Grandison and Guidotti, 1979, or Enna and Maggi, 1979) is in the retina transported into certain amacrine cells. Recent quantitative measurements (Snodgrass, 1978; Lodge, Curtis, and Johnston, 1978; Johnston, Kennedy, and Lodge, 1978) have shown that muscimol is also transported into rat CNS neurons as well as into cultured astrocytes (Schousboe, Krogsgaard-Larsen, Svenneby, and Hertz, 1978) or goldfish and chicken retinal neurons (Yazulla and Brecha, 1980) by GABA transport processes. Whatever receptor binding there might be remains undetected in our experiments since none was seen autoradiographically in experiments at 0°C or in the presence of ouabain (10^{-5} M and 10^{-6} M). The results agree very well with those recently published by Yazulla and Brecha (1980) and Pourcho (1980 a).

The present observations on GABA uptake into rabbit retina agrees with previous reports (Ehinger and Falck, 1971; Iversen and Schon, 1973; Brandon, Lam, and Wu, 1979; Lam *et al.*, 1980). In goldfish, we were not able to reproduce the striking difference in horizontal cell labelling in the dark and light adapted states that was previously reported (Lam and Steinman, 1971; Lam *et al.*, 1980). We find it particularly noteworthy that horizontal cells both will and will not show labelling in different regions in one and the same retina, irrespective of whether it has been exposed to light or not, and that there seems to be a sharp boundary between the regions with labelled and unlabelled horizontal cells. We do not know whether the regional differences in horizontal cell labelling that we have observed represent true differences in the uptake system or only differences in the efficiency with which the (^3H)-GABA reaches the horizontal cells.

After treating retinas with (^3H)-muscimol or (^3H)-nipecotic acid, radioactivity appears in the inner plexiform layer where the amacrine cells have their processes, and in cell bodies with the size and position of amacrine cells. There is therefore little reason to doubt that it is amacrine cells that become labelled even though the autoradiography lacks the resolution required to follow individual cell processes. However, both substances are evidently to a significant extent also transported into glia (as such or as metabolites) because radioactivity is also seen with the distribution typical for glia. This is not unexpected with nipecotic acid because it is a GABA uptake inhibi-

tor, apparently antagonizing both neuronal and glial transport (see, e.g., Schousboe, Thorbek, Hertz, and Krogsgaard-Larsen, 1979, or De Feudis, Ossola, Elkouby, Wolff, and Mandel, 1979). Moreover, (^{14}C)-nipecotic acid was autoradiographically shown to be taken up both in glial cells and, less, in neurons of rat spinal sensory ganglia (Minchin, 1979), much similar to the (^3H)-GABA uptake (Schon and Kelly, 1974 a and b; Iversen and Kelly, 1975). The glial uptake in rabbit and goldfish is in our hands similar in magnitude to that of (^3H)-GABA and is great enough to make both substances difficult to use as markers for GABA neurons in these species. It seems to be less disturbing with immersion fixation protocols (Yazulla and Brecha, 1980; Pourcho, 1980 a), perhaps because the substances are more readily eluted from glia than from neurons where there are protected storage sites. There may also be species variations such as previously have been noted in the GABA uptake system (Ehinger, 1972 and 1978; Voaden, 1976 and 1978; Lam *et al.*, 1980).

Isoguvacine is a GABA agonist which is reported not to inhibit (^3H)-GABA uptake (Krogsgaard-Larsen *et al.*, 1977; Krogsgaard-Larsen and Johnston, 1978; Schousboe, Krogsgaard-Larsen, Svenneby, and Hertz, 1978; Lodge, Curtis, and Johnston, 1978; Schousboe, Thorbek, Hertz, and Krogsgaard-Larsen, 1979) or to potentiate GABA agonists (Lodge, Curtis, and Johnston, 1978) and which has therefore not previously been suspected to be taken up by neurons. It binds to GABA receptors in mouse brain synaptic membranes (Morin and Westerlain, 1980). However, autoradiograms of receptor bindings usually do not demonstrate radioactivity of the intensity seen in the present study. Also, the distribution of radioactivity is similar to that of GABA neurons, with radioactivity in cell bodies. This is the result expected with neuronal uptake rather than receptor binding and we therefore suggest there is such an uptake of isoguvacine. It remains uncertain what type of neurons isoguvacine labels, even if the morphological details (most radioactivity in the innermost sublayer of the inner plexiform layer; size and approximate frequency of the radioactive perikarya) are similar to those of GABA-accumulating neurons. It may be noted that the retinal GABA transport systems have some peculiarities not seen in the brain. Retinal neurons accumulate beta-alanine (Bruun, Ehinger, and Forsberg, 1974; Bruun and Ehinger, 1974) which in other parts of the CNS is felt to be accumulated predominantly by glia (Kelly and Dick, 1976). They also accumulate different omega-amino acids (Ehinger, 1976) and isoguvacine can be regarded as a kind of omega-amino acid, sterically closely related to GABA (see Krogsgaard-Larsen, Hjerds, Curtis, Lodge, and Johnston, 1979). Another peculiarity is that DABA (diaminobutyric acid) is in brain believed to

label mainly GABA neurons (*Kelly and Dick, 1976*), but in the retina it is taken up (but not retained) also in glia (*Bauer and Ehinger, 1978*). It therefore cannot be excluded that the neuronal isoguvacine uptake is specific to the retina. It is on the other hand not very likely to be the result of metabolism of the isoguvacine because good uptake is seen already in short incubations, similar to the ones which failed to show any sign of uptake in brain tissue (*Krogsgaard-Larsen et al., 1977; Krogsgaard-Larsen and Johnston, 1978; Schousboe, Krogsgaard-Larsen, Svenneby, and Hertz, 1978; Lodge, Curtis, and Johnston, 1978; Schousboe, Thorbek, Hertz, and Krogsgaard-Larsen, 1979*).

Retinas from different animal species show striking differences in the way they handle GABA (*Ehinger, 1972*, see also reviews by *Voaden, 1976, 1978, Ehinger, 1978, and Lam et al., 1980*) and muscimol (see above). Our pilot experiments (see Introduction) also hint at species differences in the way the retina handles the GABA uptake inhibitor, (^3H)-ACHC, as do the experiments of *Cunningham, Marshall, and Neal (1980)*. We are therefore currently examining the ability of isoguvacine to label retinal neurons in other species than rabbit and experiments are also in progress with the aim to identify more precisely the cells accumulating it and to characterize the uptake mechanism. Preliminary results suggest an active uptake system which is inhibitable by GABA.

The low degree of uptake of (^3H)-isoguvacine into glia is in good agreement with previous biochemical reports (see *Schousboe, Thorbeck et al., 1979*). It clearly makes (^3H)-isoguvacine an attractive drug for neuron labelling, irrespective of whether it is accumulated in GABA neurons or in another type.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (project 14X-2321), from the T. and R. Söderbergs Stiftelse, the H. Järnhardts Stiftelse, the Thorsten and Elsa Segerfalks Stiftelse and the Faculty of Medicine, University of Lund.

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Retinal Gaba Neuron Labelling with [^3H]Isoguvacine in Different Species

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(Received 13 April 1982 and accepted 18 June 1982, London)

Retinas from goldfish, chicken, rat, guinea-pig, rabbit and humans were exposed to [^3H]isoguvacine either by intravitreal injection *in vivo* or by incubation in a balanced salt solution. The distribution of radioactivity was then studied by autoradiography. The substance labelled a set of presumed amacrine cells in all types of animals. The inner plexiform layer was well demarcated and a variable number of ganglion cells was marked. In goldfish and chicken, radioactivity could also be seen in horizontal cells, particularly when the retina had detached from the pigment epithelium. Even 24 hr after an intraocular injection there was a significant amount of radioactivity left in nerve cells. Only a little glial labelling could be seen at any time. Since the distribution of labelled neurones was similar to that of GABA neurones and since isoguvacine is a potent GABA agonist, it seems reasonable to presume that [^3H]isoguvacine labels GABA neurones. The uptake is predominantly neuronal with strong binding to presumed GABA storage sites.

1. Introduction

GABA is very likely one of the transmitter substances in the central nervous system (Curtis, 1978) including the retina (Ehinger, 1982). Very often, [^3H]GABA labelling has been used for identifying morphologically the neurones that may use GABA as a neurotransmitter. However, GABA itself is not only taken up into neurones but it also enters glial cells, disguising the neuronal labelling (Marshall and Voaden, 1974b; Neal and Iversen, 1972; Ehinger, 1977). There is therefore a need for an agent which will selectively label GABA neurones but not glia.

Experiments have been performed with β -alanine and some other ω -amino acids, but success has been restricted. β -Alanine labels some neurones in the rabbit retina (Bruun, Ehinger, and Forsberg, 1974) but in the central nervous system the glial labelling is pronounced (Kelly and Dick, 1976). Labelling with [^3H]diaminobutyric acid (DABA) was found to give a somewhat better localization than [^3H]GABA in some species but still with much glial labelling (Bauer and Ehinger, 1978), particularly in frogs and rats (Cunningham, Marshall and Neal, 1981). Muscimol, a GABA agonist (Krogsgaard-Larsen, Hjeds, Curtis, Lodge and Johnston, 1979), has been reported as a receptor labelling substance in the central nervous system (Chan-Palay, 1978) but recent experiments have shown that cellular uptake is the dominating labelling process in the retina if special precautions are not taken to block these uptake systems (Yazulla and Brecha, 1980; Pourcho, 1981; Agardh and Ehinger, 1982). Nipeccotic acid, a GABA uptake inhibitor, has likewise been found to have too high a glial uptake to be useful as a neuronal GABA marker in the retina (Agardh and Ehinger, 1982). [^3H]aminocyclohexane carboxylic acid (ACHC) was found to label neurones in frogs but only Müller cells in rats, guinea-pigs and rabbits (Cunningham, Marshall and Neal, 1981; Agardh and Ehinger, 1982). So-called chase labelling with [^3H]glutamine has

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also been tried for GABA neurone marking with only partial success (Voaden, Lake, Marshall and Morjaria, 1978).

However, recently we have discovered that isoguvacine, a selective GABA agonist, preferentially labels certain neurones in the retina, and glial cells only to a very small extent. Since the distribution of GABA neurones varies in different species (Voaden, 1978; Ehinger, 1982) several animal species have now been examined to see if the structures labelled are the same as the ones labelled by [^3H]GABA and which are likely to use GABA as their neurotransmitter.

2. Materials and Methods

Intraocular injections

Light-adapted eyes were injected intravitreally with [^3H]isoguvacine and enucleated after 30 min, 4 or 24 hr. Small pieces of the posterior segments were frozen in liquid commercial propane cooled by liquid nitrogen, freeze-dried at -35°C in a tissue freeze-drier, fixed in gaseous formaldehyde at 80°C for 1 hr, embedded in plastic (Durcopan^(R)), cut at $4\text{ }\mu\text{m}$ and covered with autoradiographic stripping film (Kodak AR 10). The procedure has been described previously (Ehinger and Falck, 1971) and has been shown to be free from diffusion or extraction artefacts for a number of amino acids at the light microscopical level. After 1–12 weeks of exposure the autoradiograms were developed and fixed. The sections were counterstained with 1% toluidine blue in 40% ethanol as required.

The doses injected were: rabbit 20 μCi , guinea-pig 10 or 20 μCi , rat 10 or 20 μCi , chicken 20 μCi and goldfish 5 or 10 μCi . At least four eyes were obtained for each species and dose.

Incubations

In standard incubations, pieces of retina from light-adapted goldfish, chicken, rat, guinea-pig, rabbit and humans were incubated for 15 or 30 min in a balanced salt solution (Ames and Nesbitt, 1981) at 37°C , except goldfish retinas which were kept at 22°C . The [^3H]isoguvacine concentration was 5 $\mu\text{Ci}/\text{ml}$. The three human eyes were enucleated because of tumours. The retina was obtained from a part well away from the lesion. After the incubation, the tissue pieces were washed twice for 5 min with the balanced salt solution at 0°C . The tissue pieces were then processed for autoradiography as described above.

Dark-adapted goldfish retinas were also incubated for 15 or 30 min with [^3H]isoguvacine (10 $\mu\text{Ci}/\text{ml}$) as described above, but in darkness. They were dissected under dim dark red illumination.

In a further series of experiments rabbit and goldfish retinas were preincubated in GABA, taurine, β -alanine or glycine, all 10^{-3} M , for 10 min before the [^3H]isoguvacine (5 $\mu\text{Ci}/\text{ml}$) was added and also during the incubation with the radioactive drug. The experiments were otherwise performed as standard incubations.

[^3H]isoguvacine was obtained from New England Nuclear Co. It was delivered at a concentration of 1 $\mu\text{Ci}/\mu\text{l}$ dissolved in 2% ethanol in water and used as such.

3. Results

In all species round or oval labelled cells were seen in the few innermost cell rows of the inner nuclear layer. Radioactivity could occasionally also be seen in pear-shaped cells with this location in chicken and guinea-pig retinas. At times, it was possible to distinguish single processes from the pear-shaped cells, reaching a sublayer in the inner plexiform layer. The labelled cells often occurred singly but at times they seemed to occur in groups of two or three cells. There was a slight difference in the degree of radioactivity in individual cells, but this difference was not as pronounced as in cells in the ganglion cell layer (see below).

Labelling was also found in ganglion cells. Most of them were of the same size as

the labelled cells seen in the inner nuclear layer and then usually also as heavily labelled. Larger ganglion cells were also occasionally labelled, but then less heavily. Unlabelled, or only weakly labelled, large ganglion cells were common. The frequencies of labelled amacrine and well- and less-well-labelled ganglion cells are given in Table 1. However, many cells have intermediate degrees of labelling. The figures are therefore subject to large errors due to the observer and should only be taken as rough estimates. The labelled ganglion cells did not cluster in groups. The pigment epithelium remained unlabelled.

TABLE I

*Frequency of labelled neurones in different species as counted in 4 μ m sections 4 hr after the intravitreal injection of [³H]isoguvacine**

Species	Amacrine cells per mm retina	N†	Well labelled cells in the ganglion cell layer		All labelled cells in the ganglion cell layer		Horizontal cells per mm retina
	$\pm$ S.E.M.		Per mm retina	%	Per mm retina	%	
Rabbit	48 $\pm$ 3	160	6 $\pm$ 1	37 $\pm$ 2	10 $\pm$ 1	61 $\pm$ 4	—
Guinea-pig	88 $\pm$ 6	236	22 $\pm$ 3	45 $\pm$ 5	25 $\pm$ 5	75 $\pm$ 5	—
Rat	80 $\pm$ 5	300	19 $\pm$ 4	29 $\pm$ 3	39 $\pm$ 3	51 $\pm$ 3	—
Chicken	181 $\pm$ 5	445	13 $\pm$ 2	13 $\pm$ 2	30 $\pm$ 6	41 $\pm$ 3	46 $\pm$ 7
Goldfish	45 $\pm$ 6	182	5 $\pm$ 2	11 $\pm$ 2	19 $\pm$ 3	38 $\pm$ 4	—

* Cells in the ganglion cell layer were scored either as well labelled or well labelled plus doubtfully labelled.

† (N) is the total number of ganglion cells counted. The values represent means $\pm$ S.E.M.

There was only a slight diffuse glial labelling which decreased with the duration of the drug in the eye. No cell bodies in the middle of the inner nuclear layer, and no radial strands characteristic for glial Müller cells could be seen.

Intraocular injections: goldfish

Both 30 min and 4 hr after the injection of 5 or 10 μ Ci [³H]isoguvacine radioactivity appeared in the inner plexiform layer, at times with a tendency for the innermost third to be more heavily labelled. Cell bodies with the position, size and shape of amacrine cells as well as ganglion cells were also marked. The frequencies of labelled cells are given in Table I.

Injecting 20 μ Ci [³H]isoguvacine into goldfish eyes resulted in strong radioactivity but no additional structures became labelled. Horizontal cells became weakly labelled, and were visible only after overexposure of the inner plexiform layer in the autoradiographs. The glial labelling was still insignificant. Unlabelled round areas appeared just inside the border of the inner plexiform layer (Fig. 1). These are interpreted as unlabelled ganglion cells or blood vessels.

After 24 hr: the inner plexiform layer and cells with the position of amacrine cells as well as ganglion cells were labelled as described above. Radioactivity could also be seen in regional areas throughout the inner nuclear layer, but the radioactivity was not localized to glial cells, which were not significantly labelled (Fig. 2).

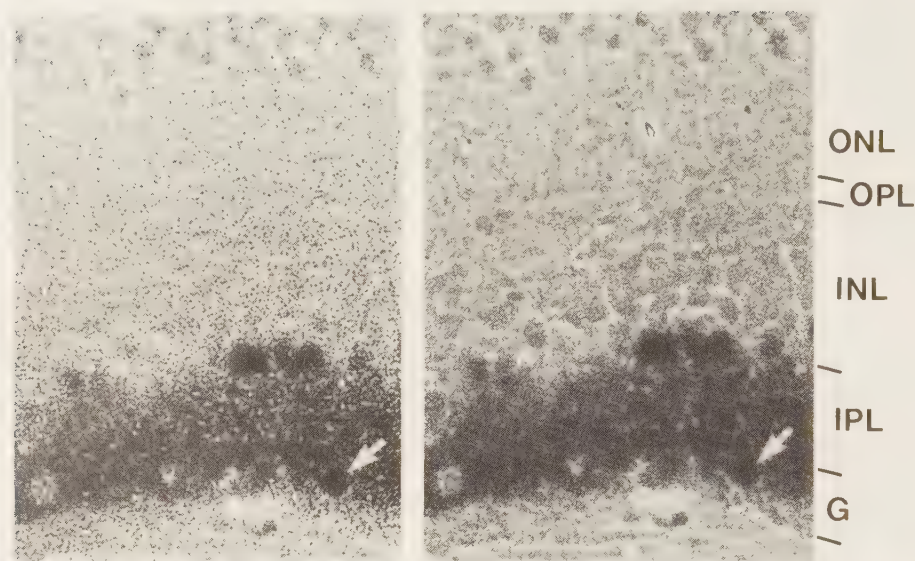


FIG. 1. Goldfish retina 4 hr after the intravitreal injection of 20 μ Ci [3 H]isoguvacine. Radioactivity appears in the inner plexiform layer, more marked in the innermost part towards the ganglion cell layer. Cells with the position of amacrine cells and one ganglion cell (arrow) are labelled. The unlabelled areas in the innermost part of the inner plexiform layer are interpreted as unlabelled ganglion cells or blood vessels. Outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (G). Left, focus on the silver grains; right, focus on the section. Phase-contrast micrograph, $\times 450$.

Incubations: goldfish

These experiments were performed on dark-adapted retinas which had gently been peeled away from the choroid and pigment epithelium. Light-adapted retinas are not readily peeled off. The same structures became radioactive as after intraocular injections, but the horizontal cells were more clearly labelled. In tangential or semitangential sections it could be seen that they were situated in the most sclerad row of the inner nuclear layer, that is they are likely to be H1 cells. There was weak labelling in the outer plexiform layer, most marked close to the labelled horizontal cells (Fig. 3). Light-adapted retinas were preincubated with certain amino acids (10^{-3} M). GABA blocked the uptake of [3 H]isoguvacine in all cell types and in the inner plexiform layer. Taurine and glycine had no effect but β -alanine partly blocked the uptake by a general diminution of radioactivity.

Intraocular injections: chicken

After 4 hr (Fig. 4) there were two densely labelled sublayers (laminae 2 and 4) in the inner plexiform layer. In some regions radioactivity also appeared more prominently in the innermost part of sublamina 5 as well. In addition, horizontal cells were marked but not as clearly as the amacrine cells. They were situated in the most sclerad row of the cells in the inner nuclear layer. The cell bodies were round, or oval, and did not appear in groups. There was a ribbon of weak radioactivity in the outer plexiform layer. Radioactive cells in the inner nuclear layer appeared in the two cell rows closest to the inner plexiform layer, but most of the labelled cells were in the innermost row.

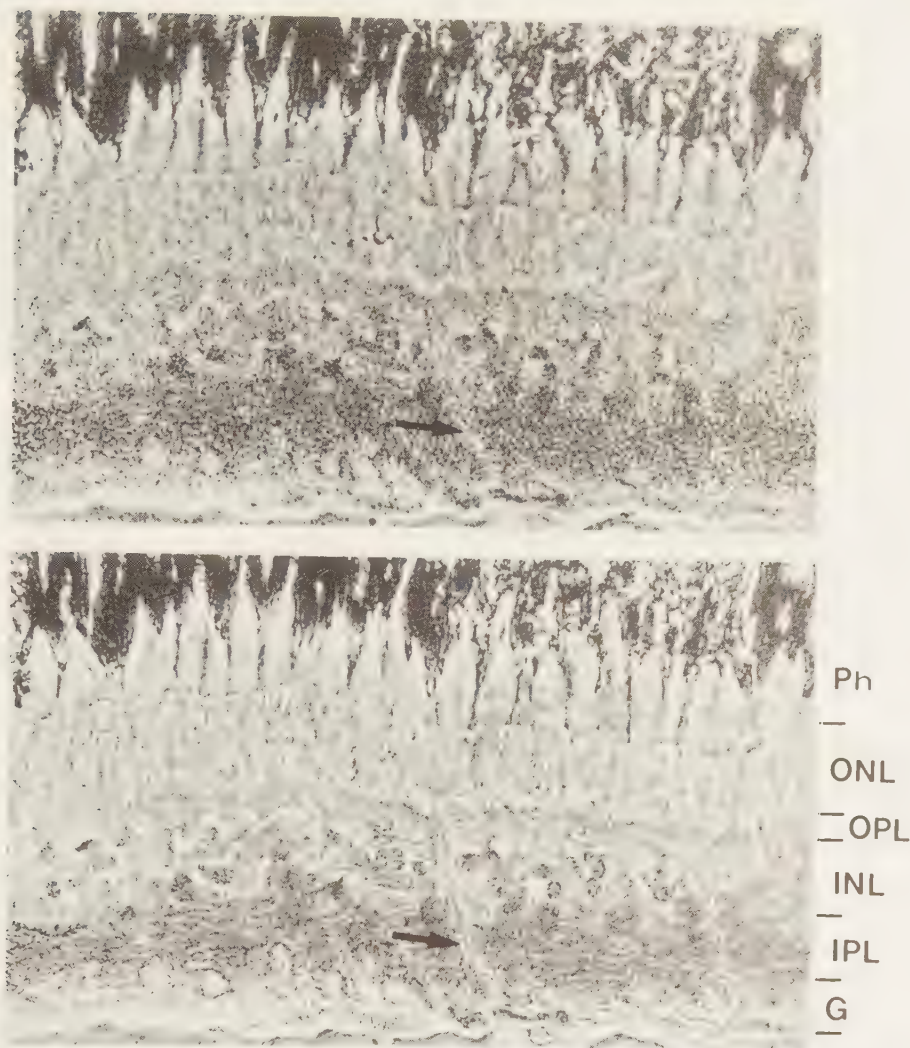


FIG. 2. Goldfish retina 24 hr after the intravitreal injection of $20\ \mu\text{Ci}$ [^3H]isoguvacine. Radioactivity appears in the inner plexiform layer, and in cells with the position of amacrine cells. The Müller cells are not significantly labelled and the section shows one such cell where this is particularly evident (arrow). Designation of layers as in Fig. 1. Above, focus on the silver grains; below, focus on the section. Phase-contrast micrograph, $\times 510$.

Radioactive ganglion cells could also be seen, but not as clearly marked as the amacrine cells. Cell counts are given in Table 1.

After 30 min the inner plexiform layer was radioactive, but without sublayers. In some regions of the sections the outer part of the inner nuclear layer was seen to be slightly more radioactive than the inner, but no labelled cell bodies corresponding to horizontal cells were visible. There was also a slight radioactivity in the outer plexiform layer. Amacrine cells and ganglion cells were labelled as described above.

Twenty-four hours after the injection of [^3H]isoguvacine the radioactivity was weaker, but still apparent, in the inner plexiform layer showing the same sublayering



FIG. 3. Goldfish retina, dark adapted, incubated in $5 \mu\text{Ci/ml}$ [^3H]isoguvacine for 30 min. Radioactivity appears in the inner plexiform layer, in cell bodies with the position of amacrine cells and in addition in horizontal cells (arrows). The outer plexiform layer is weakly labelled. Designation of layers as in Fig. 1. Dark-field illumination, $\times 410$.

as after 4 hr. Radioactivity was also still present in cells in the inner nuclear layer and weakly in the horizontal cells. The distribution and frequencies of the labelled cells were not apparently different when compared with the 4 hr experiment.

Incubations: chicken

Most of the retinas had detached. Sublayer formation was also apparent in these experiments, and with the same location as after intraocular injections but sublaminae 4 and 5 often appeared as one homogeneous layer. The amacrine cells were also labelled, but very few ganglion cells, and their distribution and morphology were identical with what was seen after the intravitreal injections. However, the horizontal cells were now very prominent and appeared as radioactive as the amacrine cell bodies (Fig. 5). The outer plexiform layer was also more intensely labelled. When the retinas were not detached the horizontal cells and the outer plexiform layer appeared less radioactive.

Intraocular injections: rat

Four hours after the injection of $10 \mu\text{Ci}$ [^3H]isoguvacine, radioactivity appeared in the inner plexiform layer, at times somewhat more markedly in its inner third than in the other parts, and also in cells in the innermost cell row of the inner nuclear layer (Fig. 6). In addition, some cells in the second cell row were labelled. They are included

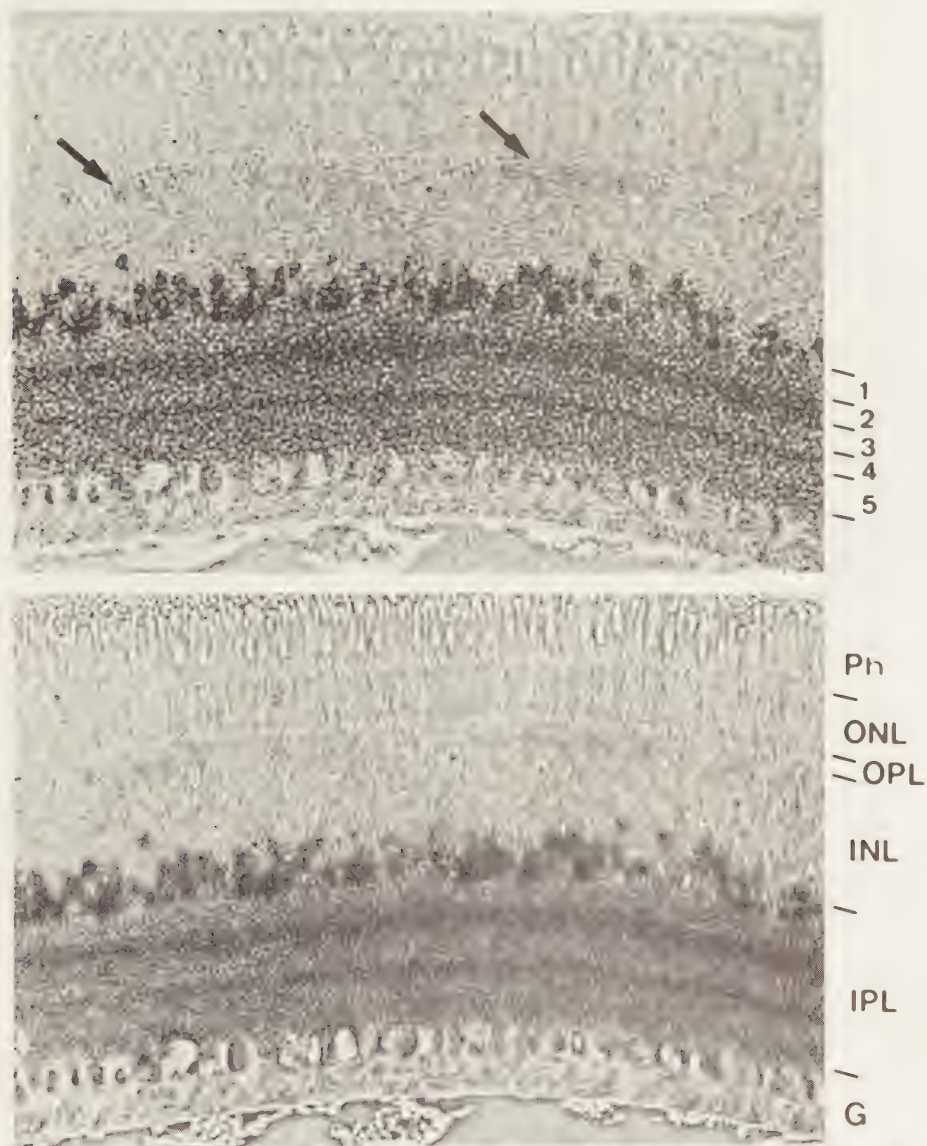


FIG. 4. Chicken retina 4 hr after the intravitreal injection of 20 μ Ci [³H]isoguvacine. Radioactivity appears in the inner plexiform layer, particularly in sublayers 2 and 4. Cell bodies with the position of amacrine cells and ganglion cells are labelled. In addition, radioactivity appears weakly in horizontal cells and in the outer plexiform layer (arrows). Designation of layers as in Fig. 1. Above, focus on the silver grains; below, focus on the section. Phase-contrast micrograph, $\times 345$.

in the frequency counts in Table I. Ganglion cells were also labelled to a variable degree.

After 30 min, the same structures were radioactive. The same type of sublayering of the inner plexiform layer as after 4 hr was already occasionally present. Also after 24 hr the same structures were radioactive, although the general level of labelling had decreased.

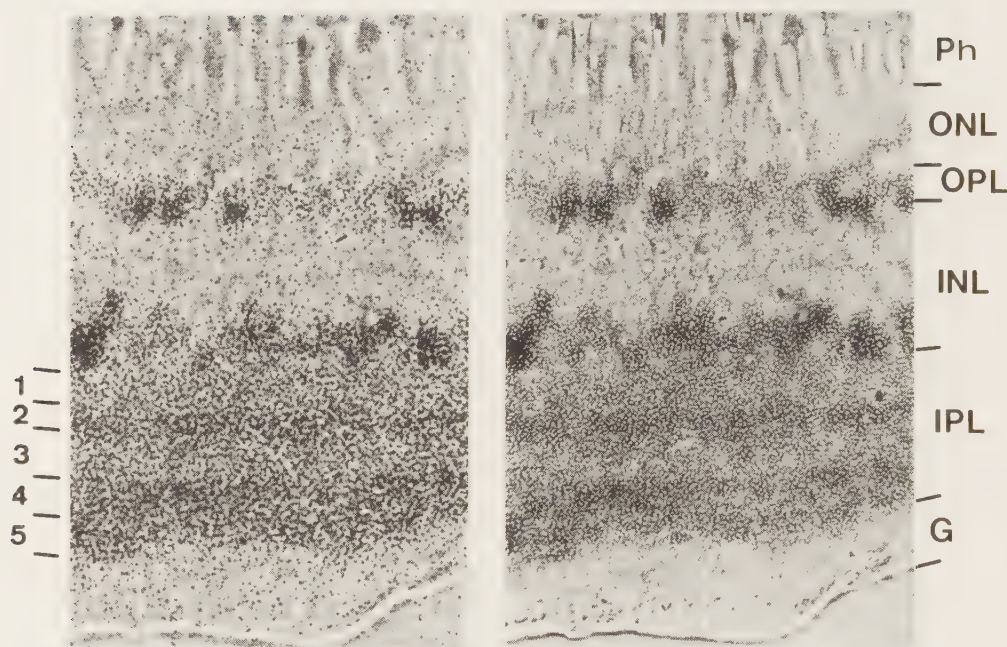


FIG. 5. Chicken retina, incubated for 15 min in $5 \mu\text{Ci/ml}$ [^3H]isoguvacine. Radioactivity appears in the inner plexiform layer, more markedly in sublayer 2 and in sublayers 4 and 5. Cell bodies with the position of amacrine cells and horizontal cells are equally labelled, and the outer plexiform layer also appears radioactive. Designation of layers as in Fig. 1. Left, focus on the silver grains; right, focus on the section. Phase-contrast micrograph, $\times 475$.

As with goldfish, the higher dose ($20 \mu\text{Ci}$) of [^3H]isoguvacine resulted in a higher general level of radioactivity, but brought out no additional structures.

Incubations: rat

In these retinas, the inner plexiform layer was labelled, more prominently in its innermost part. The cell bodies, both in the inner nuclear layer and in the ganglion cell layer were not well marked, but could be detected.

Intraocular injections: guinea-pig

Thirty minutes after an intraocular injection of [^3H]isoguvacine there were regional differences with some parts of the retina well marked and others less well marked. Radioactivity appeared in the inner plexiform layer with a tendency to form sublayers in the innermost (sublamina 5) and outermost (sublamina 1) parts (Fig. 7). In some regions it was possible to distinguish a third sublayer inbetween the other two. Its location varied between sublayer 3 and 4. Radioactivity also appeared in cells in the innermost row of the inner nuclear layer and in ganglion cells. Occasionally, processes from pear-shaped amacrine cells reached the middle of the sublayers described here.

After 4 hr the pattern was the same, but there were no longer any regional differences and the sublayer formation in the inner plexiform layer had disappeared. The frequencies of labelled neurones are given in Table I.

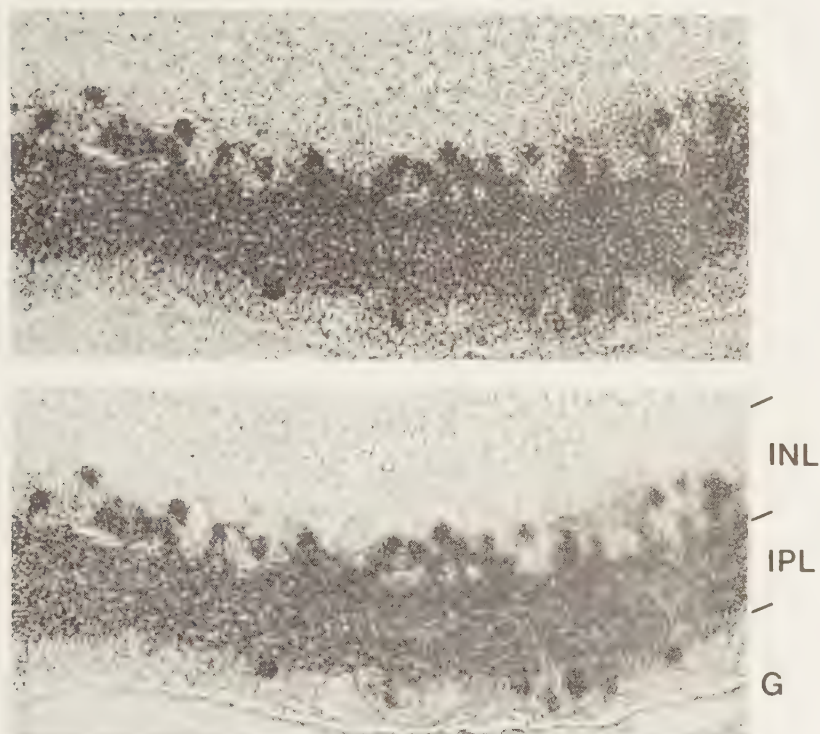


FIG. 6. Rat retina 4 hr after the intravitreal injection of 10 μ Ci [³H]isoguvacine. Radioactivity appears in the inner plexiform layer. In addition, cell bodies with the position of amacrine cells, and, to a variable degree, ganglion cells are labelled. Designation of layers as in Fig. 1. Left, focus on the silver grains; right, focus on the section. Phase contrast micrograph, $\times 360$.



FIG. 7. Guinea-pig retina 30 min after the intravitreal injection of 10 μ Ci [³H]isoguvacine. Radioactivity appears in the inner plexiform layer with a vague tendency to form sublayers in the innermost and outermost parts, and in the middle of the inner plexiform layer. Cell bodies with the position of amacrine and ganglion cells are labelled. Designation of layers as in Fig. 1. Dark field illumination, $\times 400$.

Twenty-four hours after intraocular injection radioactivity was still present, though less pronounced, in the same structures as after 4 hr.

Injection of 20 instead of 10 μCi showed only higher radioactivity but no additional labelled structures.

Incubations: guinea-pig

In these experiments the inner plexiform layer became radioactive with accumulation in the innermost part towards the ganglion cell layer. Cells in the innermost part of the inner nuclear and ganglion cell layers also became labelled but the cell bodies were not as well marked as after intraocular injection and the general level of radioactivity was lower.

Intraocular injections: rabbit

Four hours after the injection, radioactivity appeared mainly in the inner plexiform layer and in cell bodies with the position of amacrine cells in the innermost cell row of the inner nuclear layer (Fig. 8). Ganglion cells were also labelled. The frequency of labelled cells 4 hr after the injection of [^3H]isoguvacine is given in Table I.

Thirty minutes after the injections, the picture was almost the same, but there was a tendency for the delineation of a more densely labelled sublayer both in the innermost and outermost parts of the inner plexiform layer. There was a considerable variation in the degree of labelling in different pieces from the same retina, some pieces being heavily labelled and some not at all. In the most heavily labelled parts there was a discrete labelling of glial cells in addition to the neurones.

Twenty-four hours after the injection the radioactivity was less than after 4 hr, but there was still a significant amount of radioactivity in the inner plexiform layer, in the amacrine cells and in some ganglion cells. There was no apparent decrease in the incidence of labelled ganglion cells.

Incubations: rabbits

With the concentration used, retinas incubated in [^3H]isoguvacine usually showed less radioactivity than 4 hr after intraocular injections. However, the distribution of the radioactivity was the same, but there was a tendency for the appearance of a sublayer in the innermost part of the inner plexiform layer.

Autoradiograms from retinas preincubated in various substances were compared with simultaneously processed control retinas. Preincubation with GABA (10^{-3} M), blocked the uptake in both the inner plexiform layer and cells in the inner nuclear layer. Glycine (10^{-3} M), on the other hand, did not affect the uptake, whereas β -alanine (10^{-3} M) and taurine (10^{-3} M) had variable effects, depressing the uptake in some experiments and not in others.

Incubations: humans

These retinas were carefully peeled off from the pigment epithelium in daylight before the incubations. Radioactivity appeared homogeneously throughout the inner plexiform layer (Fig. 9). Cells in the innermost cell row of the inner nuclear layer had also accumulated radioactivity. They were round and of approximately the same size as the other cells in this row and appeared randomly distributed. No individual processes could be traced. Ganglion cells were occasionally labelled. There was no labelling in the layers outside the inner nuclear layer.

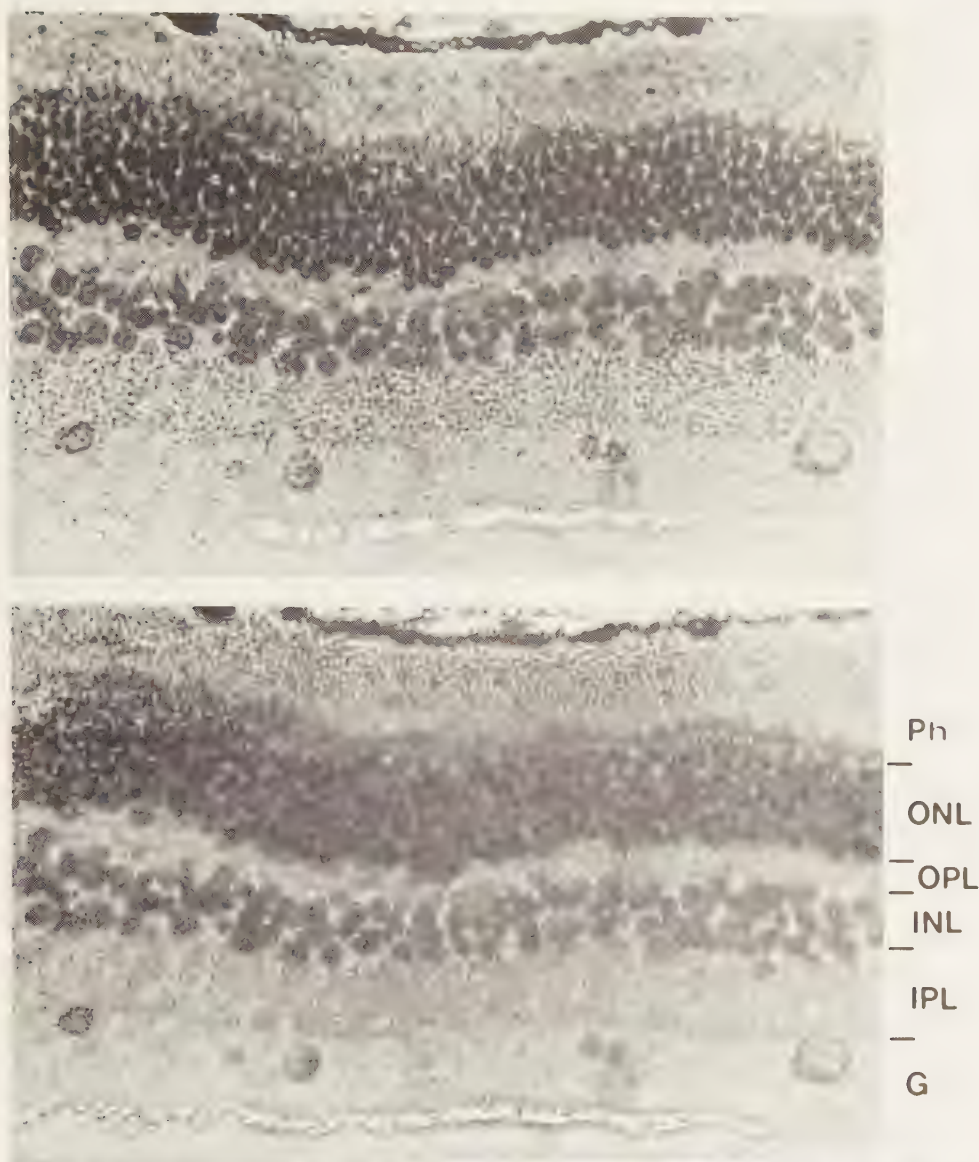


FIG. 8. Rabbit retina 4 hr after the intravitreal injection of 20 μCi [^3H]isoguvacine. Radioactivity appears in the inner plexiform layer and in cell bodies with the position of amacrine cells. Ganglion cells are labelled to a variable degree. Designation of layers as in Fig. 1. Above, focus on the silver grains; below, focus on the section. Toluidine-blue-stained micrograph, $\times 450$.

4. Discussion

In general, glial cells are characterized by having their cell bodies in the middle of the inner nuclear layer. They extend radially with comparatively broad processes, and can usually be seen as pillar like structures when radioactively labelled, as for instance after labelling them with [^3H]glutamic acid or with [^3H]GABA in certain species

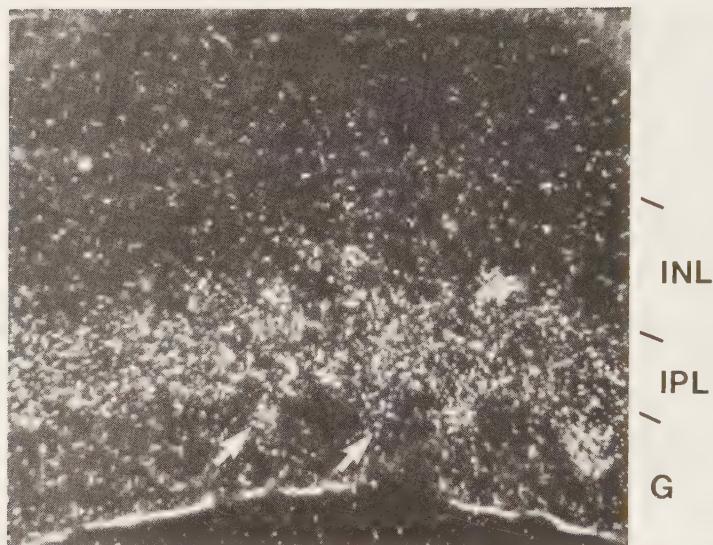


FIG. 9. Human retina, incubated for 15 min in 5 $\mu\text{Ci/ml}$ [^3H]isoguvacine. Radioactivity appears in the inner plexiform layer. Cell bodies with the position of amacrine and ganglion cells are labelled (arrows). Dark-field illumination, $\times 400$.

(Ehinger, 1977). The labelling with [^3H]isoguvacine seen in the present study was clearly different. Neurones are the only other component that is common enough to represent the labelled structures and we, therefore, conclude that the [^3H]isoguvacine labels certain neurones rather than glia.

Being a receptor agonist, [^3H]isoguvacine could conceivably label the tissue either by binding to receptor sites or by being taken up into neurones. The first alternative seems somewhat unlikely because of the high radioactivity reached in the tissue and because not only the surface of the cells but also the cytoplasm becomes labelled. Furthermore, quantitative uptake studies have demonstrated an active, uptake mechanism for [^3H]isoguvacine in the retina (Agardh and Ehinger, in prep.).

Isoguvacine resembles GABA structurally and it is therefore reasonable to hypothesize that [^3H]isoguvacine labels GABA neurones. A comparison of the distribution of labelled neurones supports this. Nerve cell bodies that label with [^3H]GABA and [^3H]isoguvacine are distributed similarly in all the species studied. Most notably, H1 horizontal cells become labelled in goldfish and they are very likely to be GABA-ergic (Marc, Stell, Bok and Lam, 1978). Horizontal cells were also labelled in chicken, which agrees very well with the [^3H]GABA labelling seen in birds (Marshall and Voaden, 1974a).

Further support for the suggestion that [^3H]isoguvacine labels GABA neurones is derived from the qualitative uptake studies. Labelling is inhibited by GABA, precisely as expected if [^3H]isoguvacine is accumulated by the same system as GABA. Glycine, which is also very likely to be a retinal neurotransmitter (Ehinger, 1982), does not affect the labelling with [^3H]isoguvacine. Quantitative uptake studies similarly show an inhibition of the [^3H]isoguvacine uptake by GABA but not by glycine (Agardh and Ehinger, in prep.) Further, we have been able to show that [^3H]isoguvacine can be released from the retina by depolarizing the neurones with potassium ions (Agardh and Ehinger, in prep.). We therefore presume that [^3H]isoguvacine labels GABA neurones in the retina.

The horizontal cells in chicken and goldfish were labelled much more in incubations

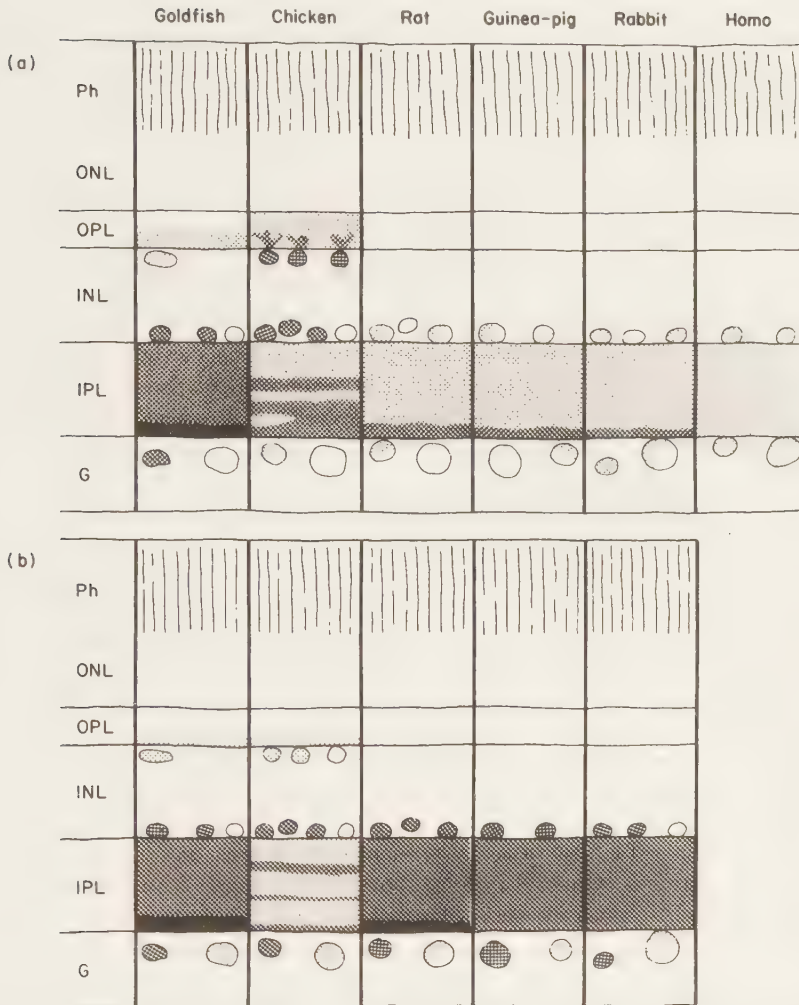


FIG. 10. (a) Schematic representation of the distribution of radioactivity after incubation for 15 min in 5 µCi [³H]isoguvacine per ml. In all species radioactivity appears in the inner plexiform layer with sublayer formation, except in humans. Amacrine cell bodies and ganglion cells are labelled, though this is less prominent in rat and guinea-pig than in the other species. In goldfish and chicken the horizontal cells and the outer plexiform layer are also labelled. (b) Schematic representation of the distribution of radioactivity 4 hr after the intravitreal injection of [³H]isoguvacine. In all species radioactivity accumulates in the inner plexiform layer with sublayers in goldfish, chicken and rat. Certain amacrine cells are evenly labelled, and ganglion cells to a variable degree. In chicken and goldfish horizontal cells and the outer plexiform layer also become radioactive.

where the retina had detached, than when it still adhered to the pigment epithelium, and in both cases much more than after intravitreal injections of [³H]isoguvacine. A similar observation has previously been made with [³H]GABA, suggesting diffusion barriers for it both in the pigment epithelium and in the inner layers of the neuroretina (Marshall and Voaden, 1974a; Hollyfield, Rayborn, Sarthy and Lam, 1979).

In goldfish no differences were apparent between detached dark and light adapted retinas which agrees with previous experiments with [³H]GABA (Agardh and Ehinger, 1982). However, Marc, Stell, Bok and Lam (1978) reported that the uptake of [³H]GABA was decreased in horizontal cells and increased in the amacrine cells in the

dark-adapted goldfish retina, whereas in frog, both horizontal and amacrine cells accumulated radioactivity (Voaden, Marshall and Murani, 1974).

The number of amacrine cells that can be labelled by [^3H]isoguvacine varies considerably between different species (Table I). The number is several times higher in chicken than in goldfish or rabbit and a sublayering is also particularly apparent in chicken. Most likely this reflects the considerably more complex construction of the inner plexiform layer of birds than many other species (Dubin, 1970). The distribution of presumed GABA neurones in different species is schematically represented in Fig. 10.

There is considerable evidence for a class of neurones in the ganglion cell layer of the retina of the rat and rabbit that do not send an axon into the brain (Perry and Walker, 1980; Vaney, Peichl and Boycott, 1981). They are usually smaller in size than the true ganglion cells, and comprise a variable proportion of the neurones in the ganglion cell layer, 32 % in rabbit retina (Vaney, Peichl and Boycott, 1981), approximately 50 % in rat and cat retina (Perry, 1981; Hughes and Wieniawa-Narkiewicz, 1980).

In our experiments the small heavily labelled neurones in the ganglion cell layer comprised between 30–45 % of all mammalian ganglion cells (Table I), suggesting they perhaps are GABA-ergic. Roughly the same proportion of cells also accumulate [^3H]choline by a high affinity, sodium-dependent uptake process (Masland and Mills, 1979; Hayden, Mills and Masland, 1980). This is usually regarded as an indicator for acetylcholine synthesizing neurones, so that it appears that the same cells may to a significant degree accumulate both GABA-ergic and cholinergic markers. The reasons for this remain unsolved.

Species differences are apparent already at the level of resolution that autoradiography provides. Sublayers appear in the inner plexiform layer in chicken and, less easily discernible, in all other species except humans. Such species variations are expected because cells with other neurotransmitters which have been analysed in more detail (such as dopamine or acetylcholine) show marked species variations both in frequency and pattern of distribution of their processes.

Because of the obscuring glial labelling, GABA neurones have not previously been identifiable in rat or human retinas. The present experiments show the usefulness of [^3H]isoguvacine for labelling presumed GABA neurones and also show that in species where they have previously not been identifiable, their morphology conforms with the general scheme seen previously.

ACKNOWLEDGMENTS

This work was supported by grants from M. Bergwalls stiftelse, C. och B. Regnérs stiftelse, Svenska Läkarsällskapet, the Faculty of Medicine, University of Lund and the Swedish Medical Research Council (Project 14X-2321).

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THE UPTAKE OF THE GABA AGONIST, [³H]ISOGUVACINE, BY THE ISOLATED RETINA OF THE RABBIT

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(Received 25 February 1983; accepted 3 May 1983)

Abstract—Previous autoradiographic studies aimed at showing neurones using GABA as their neurotransmitter have been hampered by the fact that the substance is a ubiquitous metabolite and therefore accumulated by a large variety of cells, including glia. Consequently, GABA uptake markers without this widespread uptake are desirable, and one, [³H]isoguvacine, has shown promising results in autoradiographic experiments. Its uptake has now been further studied with quantitative radiochemical techniques.

The uptake of the drug was slow compared to GABA uptake and reached a tissue/medium ratio of about 3 after 120 min. The uptake could be inhibited by GABA, beta-alanine or ouabain, and by incubating the retinas at 0°C. The uptake kinetics were complex but suggested a high affinity uptake system (K_m about 10^{-8} M) and perhaps one or several systems with lower affinities.

The results indicate that [³H]isoguvacine and [³H]GABA are accumulated and retained by the same neurones, which most likely use GABA as their neurotransmitter.

Gamma-aminobutyric acid (GABA) is a very likely inhibitory neurotransmitter in the retina (Ehinger, 1982). It is in most species also accumulated in it by an active uptake process which exhibits saturation kinetics (Voaden *et al.*, 1974; Goodchild and Neal, 1973; Neal, 1976). However, morphological studies have shown that with short incubation times, such as usually are used, there is an accumulation of radioactivity not only in neurones but also in glial and other cells (Ehinger, 1977). In many species (e.g. rat, guinea-pig and humans) it is very hard to distinguish neurones also after intraocular injections of [³H]GABA and long application times, because of the great amount of radioactivity accumulated in the glial cells. Therefore, it is of great interest to find a drug capable of selectively labelling the neurones but not the glial cells. Autoradiographic studies (Agardh and Ehinger, 1982, 1983) suggest that the GABA agonist, isoguvacine, fulfills these requirements. It gives a very distinct labelling of neurones of the same kind and distribution as GABA neurones in many species, including man (Agardh and Ehinger, 1982). The aim of this study was to characterize and quantify this isoguvacine uptake into retinal neurones.

EXPERIMENTAL PROCEDURES

Standard outbred pigmented rabbits of either sex, 1.5–2 kg, were killed by an injection of air in the ear vein. The retinas were gently peeled off from the choroid in ambient laboratory light (about 200–300 lx) and placed in 4 ml of a balanced salt solution (Ames *et al.*, 1980) at 37°C unless otherwise indicated. [³H]isoguvacine was added after 30 min to give a final concentration of 3.1×10^{-9} M and the incubations were then continued for another 30 min except when the time course was analysed. The retinas were subsequently washed at 0°C for 2×5 min in 5 ml of the balanced salt solution, weighed and homogenized in 1 ml distilled water.

The radioactivities of the incubation solutions and the retina homogenates were measured by liquid scintillation spectrometry in a commercial scintillation mixture. Quench corrections were performed by the external standard channels ratio method.

The inhibitory effects of GABA, beta-alanine, taurine (all 10^{-4} or 10^{-3} M), glycine (10^{-3} M) or ouabain (10^{-5} M) were analysed by adding appropriate amounts of these substances to the incubation medium 30 min before the [³H]isoguvacine.

The cellular localization of the uptake of [³H]isoguvacine was checked by autoradiography as previously described (Agardh and Ehinger, 1982). Briefly, the tissue pieces were freeze-dried after the incubation, embedded in plastic, cut at 4 μ and covered with Kodak AR10 autoradiographic stripping film. The [³H]isoguvacine concentration was in these experiments raised about a 100-fold in the incubation medium to 3×10^{-7} M in order not to get too long autoradiographic exposure times. This concentration is within what is usually recognized as the high affinity concentration range (less than 10^{-5} M).

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To study the uptake kinetics retinas were incubated for 10 min in a medium containing [^3H]isoguvacine (3.1×10^{-9} M) together with various amounts of non-radioactive isoguvacine (final concentrations 10^{-9} – 4×10^{-5} M).

The [^3H]isoguvacine, specific activity 40.0 Ci/mmol, was obtained from New England Nuclear Corporation and purified by paper chromatography in *N*-butanol-acetic acid-water, 25:4:20, v/v/v, to 98.7% purity.

RESULTS

Autoradiography

The distribution of radioactivity within the retina is seen in Fig. 1. Cell bodies with the position of amacrine cells were labelled, as was the inner plexiform layer. Some cells in the ganglion cell layer were also labelled. The picture is identical with that seen in earlier experiments (Agardh and Ehinger, 1982) and is therefore not dealt with in detail here.

Time course of the uptake

The tissue/medium uptake ratio was found to increase approximately linearly during the first 30–40 min, and after 120 min the system reached a tissue/medium ratio of about 3 (Fig. 2).

Effects of amino acids, ouabain and incubation at 0°C (Fig. 3)

Incubating the retinas in 10^{-4} M GABA before adding the [^3H]isoguvacine had some depressing effect on the uptake, (about 30% reduction, $P < 0.05$) and this was enhanced with 10^{-3} M GABA (about 80% reduction, $P < 0.001$). No statistically significant effect was seen with 10^{-4} M beta-alanine but with 10^{-3} M an inhibitory effect was evident (40% reduction, $P < 0.001$). The effect was less than that of 10^{-3} M GABA ($P < 0.001$). Taurine or glycine (10^{-3} M) did not affect the uptake. Ouabain (10^{-5} M) had a significant inhibitory effect, as had a lowering of the incubation temperature to 0°C ($P < 0.001$ in both cases). The results are shown in Fig. 3.

Uptake kinetics

The uptake kinetics were analysed in Lineweaver-Burk plots of measurements of uptake velocity over 30 min periods and with isoguvacine concentrations ranging from 10^{-9} to 4×10^{-5} M. The resulting curve did not show any easily recognized breakpoint between high and low affinity uptake. If regarded as a simple uptake process, a K_m of 1.93×10^{-7} M and a V_{max} of 9.52×10^{-9} mol/min/mg wet retina was obtained. However, when arbitrarily split into two ranges, 10^{-9} – 10^{-7} M and 10^{-6} – 4×10^{-5} M, K_m calculated from linear regression plots (Figs 4 and 5) gave

two different values, 6.5 ± 10^{-8} and 5.8×10^{-5} M. The corresponding values for V_{max} were 3.2×10^{-9} and 3.3×10^{-6} mol/min/mg wet retina.

DISCUSSION

Isoguvacine was previously found not to affect the [^3H]GABA uptake into rat brain slices in concentrations up to about 10^{-3} M (Krogsgaard-Larsen *et al.*, 1977; Schousboe *et al.*, 1979). The substance was therefore initially not thought to be accumulated by neurones. However, it is clear from the autoradiograms that it is indeed accumulated by neurones, in the retina mainly into amacrine cells. Only very little is taken up by glial cells. This is presumably why it

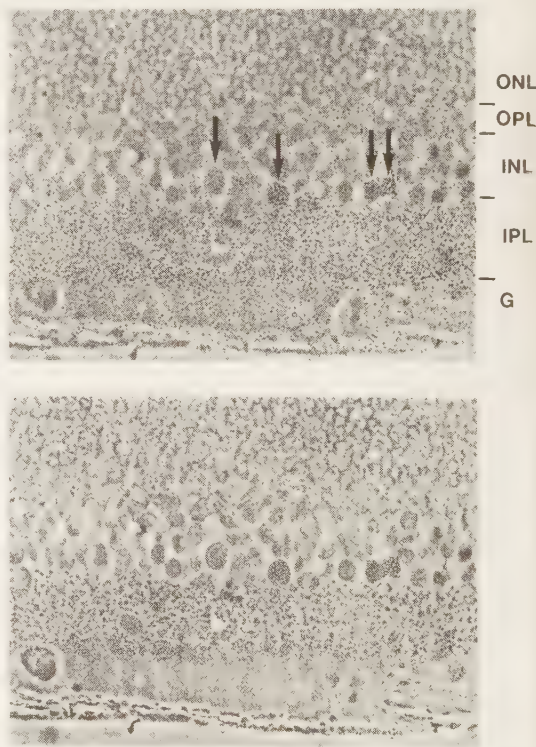


Fig. 1. Rabbit retina, incubated for 15 min in [^3H]isoguvacine, 5 μCi per ml. Radioactivity appears in the inner plexiform layer, and in cells with the position of amacrine cells. (arrows). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. Dark field illumination. Above, focus on the silver grains; below, focus on the section. Phase contrast micrograph, $\times 400$.

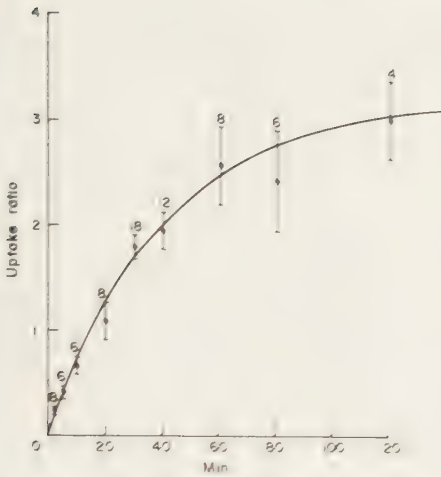


Fig. 2. Time course of [³H]isoguvacine uptake in retina incubated at 37°C. Figures indicate the number of experiments at each time. The vertical bars indicate $\pm$ S.E.M.

was difficult to see any effect on the [³H]GABA uptake, which to a great part is into glia. The observations agree with a previous report (Agardh and Ehinger, 1982), and show that the uptake measured in the quantitative experiments is into neurones, with no significant admixture of glial uptake, at least when studied with isoguvacine concentrations up to about 10^{-7} M.

[³H]Isoguvacine is not a normal constituent of the retina and is therefore most likely taken up by a sys-

tem intended for some similar, endogenous substance. Isoguvacine is a GABA agonist and has structural resemblances with this amino acid (Krogsgaard-Larsen and Falck, 1981) which suggests the possibility that it is transported by the system intended for GABA. The present experiments show that GABA inhibited the uptake of [³H]isoguvacine which is as expected if they have a common carrier. Previous and present experiments have also shown that [³H]isoguvacine is taken up by amacrine cells, and such cells in rabbits are known to accumulate and retain GABA (Ehinger and Falck, 1971; Ehinger, 1978).

Apart from [³H]GABA, amino acids also known to be accumulated by amacrine cells include [³H]glycine, [³H]taurine and [³H]beta-alanine (Bruun *et al.*, 1974; Ehinger, 1978). However, the [³H]isoguvacine uptake was not measurably affected by glycine or taurine, and it is therefore unlikely that these substances have a transport system in common with isoguvacine. Beta-alanine, on the other hand, did have some effect on the uptake, suggesting a common factor in the uptake system. However, beta-alanine is not recognized as a true neurotransmitter. It is structurally related to GABA, and available evidence suggests it is transported by the GABA system in the retina (Bruun *et al.*, 1974). It is known as a glial cell marker in some parts of the brain like the cerebellum, (Kelly and Dick, 1976; Hösli and Hösli, 1978, 1980), but this is not the case in the spinal cord, the brain stem and the retina, where it is accumulated also by neurones (Bruun *et al.*, 1974; Hösli and Hösli, 1980).

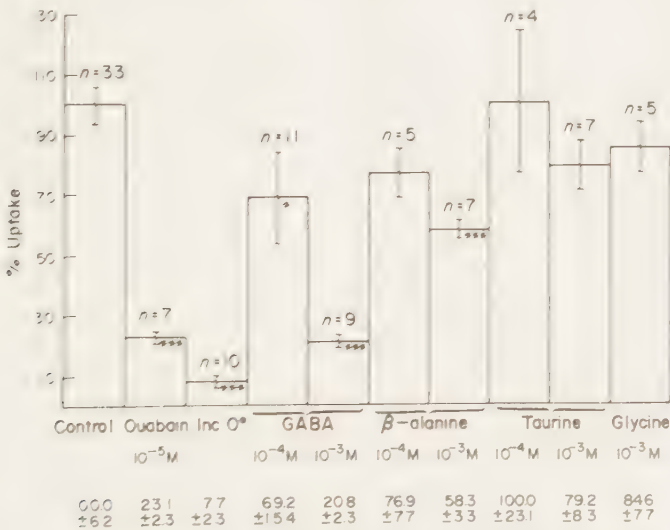


Fig. 3. Effects of ouabain incubation at 0°C and of amino acids on the uptake of [³H]isoguvacine. Vertical bars indicate $\pm$ S.E.M. *** P < 0.001; ** P < 0.01; * P < 0.05 in Student's paired two-tailed test.

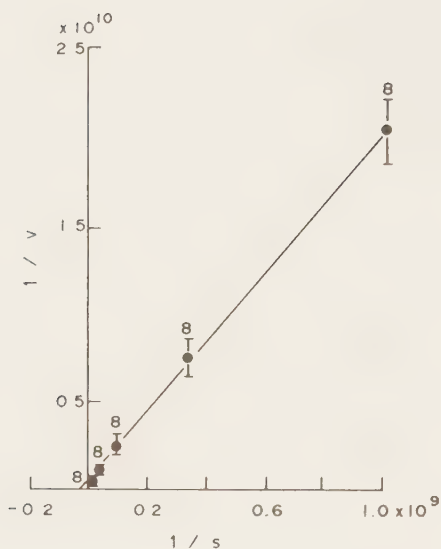


Fig. 4. Kinetic analysis of the effect of isoguvacine concentration on the rate of [^3H]isoguvacine uptake. v = rate of isoguvacine uptake, s = isoguvacine concentration (10^{-9} – 10^{-7} M). Figures indicate the number of experiments for each concentration; vertical bars indicate $\pm$ S.E.M.

The uptake systems for presumed neurotransmitters are in general active, energy dependent, high affinity systems. The uptake of [^3H]isoguvacine was strongly inhibited by lowering the incubation temperature to 0°C or by adding ouabain (10^{-5} M), a drug which blocks energy supplies by inhibiting the Na^+ – K^+ -activated ATPase. It consequently appears that the [^3H]isoguvacine is accumulated into the cells by an active, energy requiring system with properties similar to the uptake systems for neurotransmitters.

High and low affinity uptake systems are commonly distinguished by finding a breakpoint in the Lineweaver-Burk plot and then determining the Michaelis-Menten parameters by applying linear regression analysis to the segments of the curve on either side of the breakpoint. We were unable to find any easily recognizable such breakpoint in the plot and therefore arbitrarily divided the curve into two segments, one with the higher and one with the lower isoguvacine concentrations. Calculations of the Michaelis-Menten parameters for the two segments gave K_m values as if there were one system with a K_m around 10^{-8} M and another with a lower affinity, K_m around 10^{-5} M. A pilot experiment with 10^{-4} to 4×10^{-4} M isoguvacine yielded a K_m around 4×10^{-4} M. We wish to emphasize that these K_m values (and the corresponding $V_{\max}$ figures) do not have any other relevance than showing that most

likely there is in the rabbit retina a high affinity uptake system and perhaps also one or several with lower affinities for [^3H]isoguvacine.

Because we were unable to see any clear breakpoint in the Lineweaver-Burk plot, there is most likely more than two uptake systems or a considerable overlap between the high and low affinity systems. Also, we cannot exclude more complicated explanations. The K_m and $V_{\max}$ figures must therefore not be taken to represent true values for well defined systems and they are not comparable with, e.g. the K_m figures for GABA given by Neal (1976) which, furthermore, are likely to represent glial rather than neuronal uptake.

We have previously shown that there is in rabbits and several other species a considerable morphological similarity between neurones accumulating [^3H]GABA and the ones accumulating [^3H]isoguvacine (Agardh and Ehinger, 1982, 1983). The frequencies of neurones accumulating [^3H]GABA or [^3H]isoguvacine are the same. This, together with the quantitative measurements performed in the present study makes it reasonable to assume that [^3H]isoguvacine is using the GABA transport systems. Since it is not accumulated by glia to any significant extent it seems to be a useful marker of GABA neurones in the retina.



Fig. 5. Kinetic analysis of the effect of isoguvacine concentration on the rate of [^3H]isoguvacine uptake. v = rate of isoguvacine uptake, s = isoguvacine concentration (10^{-6} – 4×10^{-5} M). Figures indicate the number of experiments for each concentration; vertical bars indicate $\pm$ S.E.M.

The uptake of [³H]isoguvacine expressed as tissue to medium ratio was low, around 3 after 120 min, compared to the uptake of GABA which already after 15 min is about 12 (Neal, 1976; Ehinger unpublished). However, autoradiographical experiments have shown that incubating rabbit retinas in [³H]GABA for 15 min *in vitro* gives a predominantly glial uptake (Ehinger, 1977). Since so many more cells will accumulate [³H]GABA than [³H]isoguvacine, it is hardly surprising that uptake figures for [³H]isoguvacine are much lower than the figures for [³H]GABA. For studies on the neuronal GABA uptake system in the retina, [³H]isoguvacine would seem much more useful than [³H]GABA with which measurements are likely to become compounded by the glial uptake.

Acknowledgements—This study was supported by grants from the Faculty of Medicine, University of Lund, from the Svenska Läkarsällskapet, from Thorsten and Elsa Segerfalks Stiftelse, from Carmen and Bertil Regnérs fond and from the Swedish Medical Research Council (project 14X-2321).

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The release of (^3H)-GABA from the rabbit retina

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AGARDH, E. & BAUER, B.: The release of (^3H)-GABA from the rabbit retina. *Acta Physiol Scand* 1984, 122: 85–92. Received 30 Jan. 1984. Accepted 28 Feb. 1984. ISSN 0001-6772. Department of Ophthalmology, University of Lund, Sweden.

The neuronal and glial release of (^3H)-GABA from rabbit retina has been studied. The results indicate, that neither are there any glutamate, aspartate or glycine receptors on the GABA accumulating neurons, nor any GABA autoreceptors. (^3H)-GABA was found to be released by 40 mM K^+ from retinal neurons, but not from glia, and the release was not dependent on extracellular Ca^{++} . This indicates a release from a non vesicular transmitter pool. Ouabain has been proposed as a pharmacological tool for studying the release of (^3H)-GABA located in neuronal cytoplasm. However, it induced an increased release of (^3H)-GABA from both neurons and glia and it is therefore unlikely that it can be used for the specific purpose of studying release from neuronal cytoplasm.

Key words: Rabbit retina, GABA, GABA agonists, GABA release, receptors

Gamma-amino-butyric acid (GABA) is a likely neurotransmitter in the retina (Ehinger 1982), but details about its physiological functions are not well known. Analysing the contacts of the GABA neurons is one way of revealing their function, and we have therefore tried to characterize some receptors on GABA accumulating neurons and factors affecting the GABA release.

Glutamate and aspartate are likely excitatory transmitters in the central nervous system (Watkins & Evans 1981), and are also good candidates as transmitters in at least some parts of the retina (Miller et al. 1982). GABA and glycine are likely to be neurotransmitters in certain amacrine cells (Ehinger 1982). The first goal of this study was therefore to examine to what extent the putative neurotransmitters, glutamate, aspartate, GABA and glycine, could influence the release of (^3H)-GABA from rabbit retina by acting on a receptor, presynaptic to the GABA accumulating neurons.

Previous studies have shown a calcium-dependent, light-driven release of (^3H)-GABA from the intact rabbit retina (Bauer 1978). In synaptosomes of rabbit retina, on the other hand, both a calcium-dependent and a calcium-independent release have been found (Redburn et al. 1976, Redburn 1977), and recent studies have more directly shown a calcium independent, release of (^3H)-GABA from

horizontal cells in goldfish and in toad retinas (Schwartz 1982, Yazulla 1983, Yazulla & Kleinschmidt 1983). Such a release may have important functions different from the classical calcium dependent release. The second goal of this study was therefore to characterize the release of (^3H)-GABA from a different set of cells, the amacrine cells in the rabbit retina, and to examine to what extent it could be induced also in the absence of calcium ions.

Some of the experiments are based on the fact that in rabbits, (^3H)-GABA can be made to label predominantly either certain amacrine neurons or glial cells. Four h after an intravitreal injection of (^3H)-GABA, amacrine cells are radioactive (Ehinger 1977). Most of this radioactivity represents unmetabolized GABA (Bauer 1978). With a shorter exposure time, 30 min, the radioactivity resides mainly in glial cells (Ehinger 1977). Thus, the effects on neurons can be distinguished from those on glial cells by varying the exposure time to (^3H)-GABA.

MATERIALS AND METHODS

Pigmented rabbits of either sex were injected intravitreally with 20 μCi (^3H)-GABA. After 30 min or 4 h, the animals were sacrificed with an embolus of air intravenously. The injected eye was enucleated and the anterior

Table 1. Percentage increased release of (^3H)-GABA compared with a control curve

p indicates the statistical difference between the induced release and a spontaneous efflux of (^3H)-GABA calculated with Mann-Whitney's two-tailed ranking test. Therefore, range rather than mean values with standard errors of the mean are presented in this table. (M) = Mol, r = range, m = mean value, n = number of experiments, t = time after the intraocular injection of (^3H)-GABA

Spontaneous efflux	4 h		r=(-10) to 8	(-1.4)	5	
Spontaneous efflux	½ h		r=(-16) to 0	(-12)	5	
	t	Conc. (M)	% increased release	Mean	n	<i>p</i>
Glutamate	4 h	10^{-4}	None		5	
Aspartate	4 h	10^{-4}	None		6	
Kainic acid	4 h	10^{-5}	None		4	
Quisqualic acid	4 h	10^{-5}	None		4	
N-methyl-D-aspartate	4 h	10^{-5}	None		4	
APB	4 h	10^{-7}	None		4	
APB	4 h	10^{-6}	None		2	
APB	4 h	10^{-5}	None		4	
GABA	4 h	10^{-4}	r=9 to 56	28.5	6	<0.01
Glycine	4 h	10^{-4}	None		4	
Isoguvacine	4 h	10^{-4}	None		4	
Isoguvacine	4 h	10^{-3}	None		8	
Muscimol	4 h	10^{-4}	None		5	
Dihydromuscimol	4 h	10^{-4}	None		4	
THIP	4 h	10^{-4}	None		6	
Beta-alanine	½ h	10^{-4}	None		4	
Beta-alanine	4 h	10^{-4}	None		6	
Beta-alanine	4 h	10^{-3}	None		5	
Nipecotic acid	½ h	10^{-4}	None		4	
Nipecotic acid	½ h	10^{-3}	r=27 to 70	44	5	<0.01
Nipecotic acid	4 h	10^{-4}	None		4	
Nipecotic acid	4 h	10^{-3}	None		7	
K ⁺	½	40×10^{-3}	None		4	
K ⁺	4 h	40×10^{-3}	r=4 to 203	75.9	9	
Ouabain	4 h	2×10^{-5}	r=66 to 144	101	6	<0.01
Ouabain	½ h	2×10^{-5}	r=(-6) to 110	34.7	7	<0.01

segment and the vitreous were carefully removed. The posterior segment was inverted and placed in a water jacketed superfusion chamber as previously described (Bauer 1977). The retina was then superfused in the dark at 37°C with 1 ml/min of a balanced salt solution (Ames III and Nesbett 1981) containing 10^{-5} M amino-oxyacetic acid (AOAA) to inhibit the metabolism of GABA. The superfusion solution was aerated with a gas mixture containing 95% oxygen and 5% carbon dioxide, and the retina was left to equilibrate for 30 min in the dark before the start of the actual experiment.

Four types of experiments were performed. In the first set, an excitatory transmitter or agonist was added to the superfusion medium for 2 min while monitoring the (^3H)-GABA release. The substances tested were glutamate and aspartate (10^{-4} M), the glutamate receptor agonists kainic acid (10^{-5} M), quisqualic acid (10^{-5} M) and 2-amino-phosphono-butyric acid (10^{-7} – 10^{-5} M) as well as the aspartate receptor agonist, N-methyl-D-aspartate (10^{-5} M).

In the second set of experiments, the effects of GABA and glycine (10^{-4} M) and the effects of the GABA agonists, isoguvacine (10^{-4} or 10^{-3} M), muscimol, dihydro-

muscimol or THIP (all 10^{-4} M) were examined. To further characterize an effect on a presumed GABA autoreceptor we studied if isoguvacine, muscimol, THIP or GABA (all 10^{-6} M), could influence a potassium-induced release of (^3H)-GABA. These substances were added to the superfusion medium 5 min before a 2 min exposure to 40 mM K⁺. The experiments were performed 4 hours after the intraocular injection of (^3H)-GABA.

The effects of the uptake inhibitor nipecotic acid (10^{-3} and 10^{-4} M), and of beta alanine (10^{-3} and (10^{-4} M), on the (^3H)-GABA release were also examined both ½ and 4 h after the intravitreal injection of (^3H)-GABA.

In a third set of experiments the effects of potassium on the GABA release were studied more in detail, both ½ h or 4 h after the intravitreal injection of (^3H)-GABA. In the first case, glial cells are labelled, and in the second case, predominantly neurons (Ehinger 1977). The potassium concentration in the superfusion medium was raised by 40 mM for 2 min. The effect of this increase in the potassium concentration on the neuronal release was also studied either in a low concentration of calcium ions (0.2 mM Ca⁺⁺ and 20 mM Mg⁺⁺ with a compensatory reduction of Na⁺ to maintain osmolality) or while superfusing with

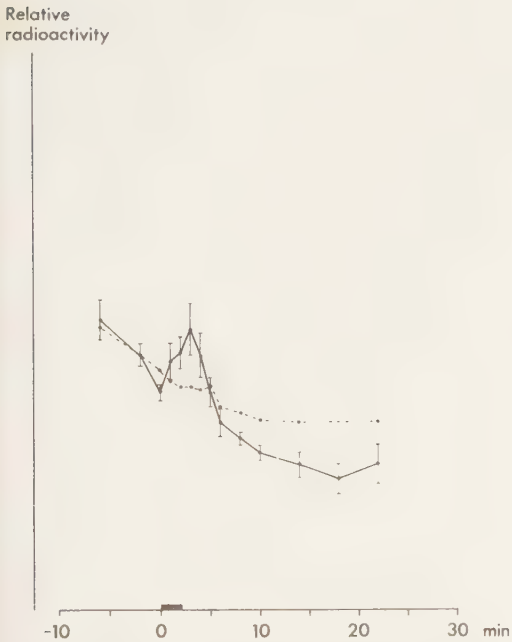


Fig. 1. The effect of GABA (10^{-4} M) on rabbit retinas exposed to (^3H)-GABA for 4 h. SE are indicated by vertical bars; 6 experiments. The dotted line represents the spontaneous efflux of (^3H)-GABA.



Fig. 2. The effect of K^+ (40 mM) on rabbit retinas exposed to (^3H)-GABA for 4 h. SE are indicated by vertical bars; 9 experiments. The dotted line represents the spontaneous efflux of (^3H)-GABA.

a medium containing no calcium at all and with 0.1 mM EGTA added to bind tissue calcium.

In a fourth set of experiments we studied the effects of ouabain (2×10^{-5} M), both $\frac{1}{2}$ and 4 h after the intravitreal injection of (^3H)-GABA.

One min superfusate samples were collected (each containing 1 ml) and 500 μl of each was added to a commercial scintillation liquid and the radioactivity was determined in a scintillation spectrometer. Quench corrections were performed with the external standards channel ratio method. The efflux curves were normalized with the aid of a computer. The (^3H)-GABA release during 5 min preceding the application of the substances tested served as a reference for the normalization. Changes in the total amount of release were compared by calculating the increase of the release in the normalized curve at a given time and comparing it with the same time in the normalized control curve, using Mann-Whitney's two-tailed ranking test.

All experiments were performed at least four times, usually more. The retina was studied 4 h or $\frac{1}{2}$ hour after the intravitreal injection of (^3H)-GABA.

The (^3H)-GABA was obtained from New England Nuclear Co. Isoguvacine, THIP and dihydromuscimol were personal gifts from Dr Krosgaard-Larsen, Copenhagen, Denmark. APB and aspartate were obtained from Calbiochem. Co., GABA and glutamate from Merck, muscimol

from CIBA-Geigy, nipecotic acid from Abbot Laboratories and the rest of the substances from SIGMA.

RESULTS

Excitatory amino acids and agonists

When retinas preloaded with (^3H)-GABA for 4 h were exposed to glutamate or aspartate, the release of radioactivity was not affected. Neither the agonists, kainic acid, quisqualic acid, N-methyl-D-aspartate, nor APB induced any increase of the rate of release of radioactivity (Table 1).

Inhibitory amino acids and agonists

GABA induced an increase of the radioactivity released from the retina preloaded with (^3H)-GABA for 4 h, ($p < 0.01$, Table 1; Fig. 1.) and this release was the same also in a medium with 0.2 mM Ca^{++} and 20 mM Mg^{++} . Glycine, on the other hand, had no effect. None of the GABA agonists, isoguvacine, muscimol, dihydromuscimol or THIP, had any effect on the release of radioactivity.

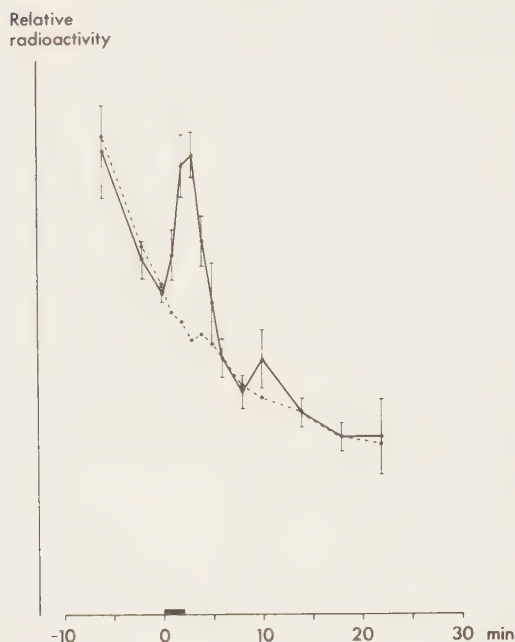


Fig. 3. The effect of nipecotic acid (10^{-3} M) on rabbit retinas exposed to (^3H) -GABA for half an hour. SE are indicated by vertical bars; 5 experiments. The dotted line represents the spontaneous efflux of (^3H) -GABA.

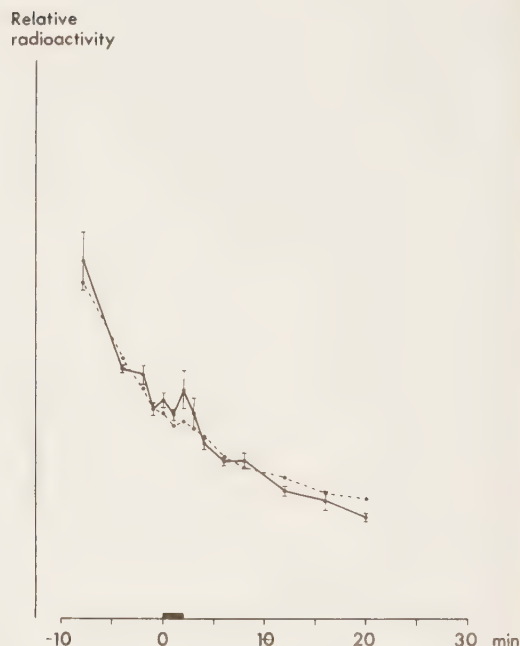


Fig. 4. The effect of K^+ (40 mM) on rabbit retinas exposed to (^3H) -GABA for $\frac{1}{2}$ h. SE are indicated by vertical bars; 4 experiments. The dotted line represents the spontaneous efflux of (^3H) -GABA.

Since 40 mM K^+ induced a release of radioactivity from the retina 4 h after the intravitreal injection ($p < 0.01$, Table 1; Fig. 2), we studied the effects of isoguvacine, muscimol, THIP and GABA, on the K^+ -induced release of (^3H) -GABA. None of these agonists were found to have any statistically significant effect (Table 2).

Beta-alanine did not cause any increased release $\frac{1}{2}$ h or 4 h after the intravitreal injection of (^3H) -GABA (Table 1). Nipecotic acid (10^{-4} M) caused no significant release of radioactivity, neither $\frac{1}{2}$ h nor 4 h after the intravitreal injection. However, by increasing the concentration to 10^{-3} M, there was a significant release from retinas exposed to (^3H) -

Table 2. Percentage increased release of (^3H) -GABA compared with a control curve

p indicates the statistical difference between the release induced by 40 mM K^+ and the composition of the superfusing medium listed below, calculated with Mann-Whitney's two-tailed ranking test. Therefore, range rather than mean values with standard error of the mean are presented in this table

	% increased release	Mean	n	p
40 mM K^+	$r = 4$ to 203	75.9	9	—
Isoguvacine 10^{-6} M + 40 mM K^+	$r = (-6)$ to 51	14.3	6	< 0.02
Muscimol 10^{-6} M + 40 mM K^+	$r = (-8)$ to 165	25	11	NS
THIP 10^{-6} M + 40 mM K^+	$r = (-24)$ to 70	29.7	7	NS
40 mM K^+ in medium (0.2 mM Ca^{2+} , 20 mM Mg^{2+})	$r = 3$ to 46	26.6	6	NS
40 mM K^+ in medium (no Ca^{2+} , 0.1 mM EGTA)	$r = (-2)$ to 34	17.4	7	< 0.1

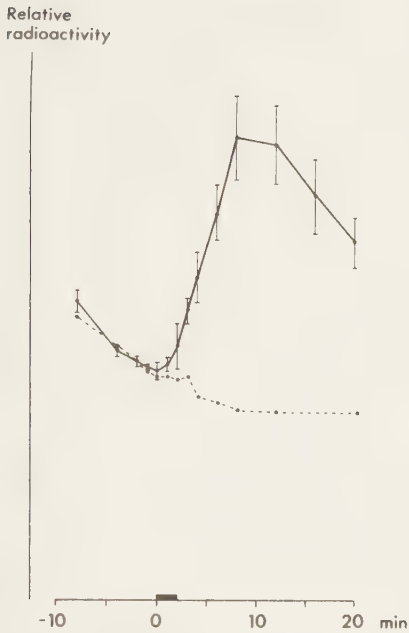


Fig. 5. The effect of ouabain (2×10^{-5} M) on rabbit retinas exposed to (^3H) -GABA for 4 h. SE are indicated by vertical bars; 6 experiments. The dotted line represents the spontaneous efflux of (^3H) -GABA.

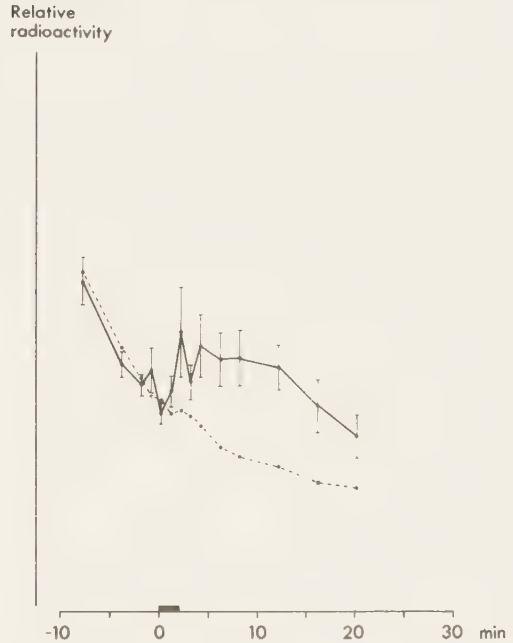


Fig. 6. The effect of ouabain (2×10^{-5} M) on rabbit retinas exposed to (^3H) -GABA for $\frac{1}{2}$ h. SE are indicated by vertical bars. The dotted line represents the spontaneous efflux of (^3H) -GABA.

GABA for $\frac{1}{2}$ h ($p < 0.01$, Table 1; Fig. 3) but no significant increase from retinas exposed for 4 h (Table 1).

Effects of potassium

Depolarizing with 40 mM K^+ caused a significant increase in the release of radioactivity from retinas with predominantly neuronal labelling ($p < 0.01$, Table 1; Fig. 2) but none from retinas with glial labelling (Table 1; Fig. 4). The neuronal release was not significantly affected by lowering the Ca^{++} concentration in the superfusing medium (Table 2). By totally excluding Ca^{++} from the medium and in the presence of 0.1 mM EGTA, there was still a statistically significant release of (^3H) -GABA ($p < 0.02$) and this release was not diminished compared with the release in the presence of Ca^{++} ($p < 0.1$; Table 2).

When ouabain was added to the superfusing medium for 2 min there was likewise a significant increase in the release ($p < 0.01$; Table 1; Fig. 5 and 6). This increase was slower in onset and longer in

duration than the K^+ -induced increase and was evident both $\frac{1}{2}$ h and 4 h after the intravitreal injection of (^3H) -GABA. A pilot experiment showed, that the retina could still respond to K^+ after the treatment with ouabain indicating that the dose used was not toxic to the retina.

DISCUSSION

Both aspartate and glutamate are neurotransmitter candidates in the plexiform layers (Altschuler et al. 1982, Miller et al. 1982, Mitchell & Redburn 1982). In a previous study (Bauer 1977), glutamate induced an increased release (^3H) -glycine from rabbit retina. We therefore expected glutamate and/or aspartate to affect the release of (^3H) -GABA from the retina. However, the study showed no such effect. One could perhaps argue that the excitatory effect of the amino acids could be counteracted by a precisely balancing inhibitory neuron, also excited by the amino acid. Several different glutamate/aspartate receptors have been demonstrated in

the retina (Mitchell & Redburn 1982) and it seems unlikely that for every such receptor possibly present on the GABA neuron, there would be a matching receptor, activating a precisely balancing inhibitory neuron as well. We therefore tested a number of selective acidic amino acid agonists (quisqualic acid, kainic acid, N-methyl-D-aspartate, and 2-aminophosphono-butyric acid, but no effect was observed with these. We therefore conclude that, most likely there are no glutamate or aspartate receptors on the GABA accumulating neurons.

Kamada et al. (1981) obtained a somewhat different result in rat retina. They found a release induced by (10^{-4} M kainic acid or 2×10^{-4} M glutamic acid (but not by 2×10^{-4} M aspartic acid). However, their labelling procedure is known to mark predominantly glia (Neal & Iversen 1972, Bruun & Ehinger 1974) and the release may therefore be an effect on glia rather than on neurons. In our study, the label was predominantly in neurons, which explains the difference in the observations. Glutamate was recently also reported to release (^{14}C)-GABA from chick retina (Morán & Pasantes-Morales 1983). In this species, the glial labelling is less pronounced than in rats (Marshall & Voaden 1974). However, the effect was shown only with very high concentrations of glutamic acid (2×10^{-3} M) and not at all in a synaptosome preparation. In the chicken retina, spreading depression can be induced by glutamate in this concentration (van Harreveld 1977) which makes it difficult to interpret the GABA releasing effect of 2×10^{-3} M glutamate.

In rat brain, Brennan et al. (1981), and Arbilla et al. (1979) have reported that GABA inhibits the release of (^3H)-GABA from GABAergic nerve endings, indicating the existence of GABA autoreceptors. We therefore tested the effects of GABA and of the GABA receptor agonists, isoguvacine, muscimol and THIP. None of them was able to inhibit the K^+ -induced release of (^3H)-GABA. Thus, our test system failed to demonstrate any GABA receptors located presynaptically to the GABA accumulating neurons.

GABA is usually regarded as an inhibitory neurotransmitter, but there are also reports of an excitatory effect (Ryall 1975). The increased release of (^3H)-GABA induced by GABA is, however, not likely to be due to an excitatory action on receptors but rather to a homoexchange, because the GABA agonists, isoguvacine, THIP, dihydromuscimol and muscimol, had no such effect.

Autoradiographic studies have indicated that the GABA analogues, isoguvacine and muscimol, accumulate in retinal neurons (Yazulla & Brecha 1980, Agardh & Ehinger 1982, 1983a, Blanks & Roffler-Tarlov 1982). Uptake studies on isoguvacine (Agardh & Ehinger 1983b) indicate that this drug is using the same uptake system as GABA, but the uptake is considerably less. It is therefore remarkable that none of these GABA analogues induced any increase of the radioactivity released from the retina. Apparently, they do not enter the same intracellular pool as GABA does. Similarly, White & Snodgrass (1983) noted that the heteroexchange of isoguvacine for GABA in rat brain synaptosomes was less pronounced than the heteroexchange of GABA for isoguvacine.

(^3H)-Beta-alanine can be released from rabbit retina by GABA (Bauer & Ehinger 1977). In this study, it did not cause any increased release of (^3H)-GABA from the retina at neither $\frac{1}{2}$ h, nor 4 h after the intravitreal injection (^3H)-GABA. This indicates, first, that it is not using the GABA transport system in glia because 30 min after the intravitreal injection, (^3H)-GABA is localized mainly in the glia (Ehinger 1977). Second, it is not taken up into the GABA accumulating neurons to any significant extent because 4 h after the intravitreal injection, (^3H)-GABA is mainly localized in neurons (Ehinger 1977). Instead, the present experiments suggest it gets localized in a pool different from that of (^3H)-GABA.

The GABA uptake inhibitor, nipecotic acid (Johnston et al. 1976), has in the retina in autoradiographic studies been shown to accumulate mainly in glia (Agardh & Ehinger 1982). Hence, it was not surprising to find that half an hour after the intravitreal injection of (^3H)-GABA, nipecotic acid caused an increased release of the radioactivity, whereas 4 h after the injection, no statistically significant increase could be noted. Apparently, nipecotic acid inhibits glial uptake rather than neuronal uptake in the retina. This is different from its action in the brain (Brehm et al. 1978).

Potassium (40 mM) was in these experiments never found to induce an increase in the release of (^3H)-GABA when the label was in glia (Fig. 4) but only when it was in amacrine neurons (Fig. 2). The suggestion (Sellström & Hamberger 1977) that such a release can take place in CNS tissue is thus not applicable to the retina under the conditions of our experiments.

A Ca^{++} -independent potassium-induced (^3H)-GABA release was previously noted in rabbit retinal synaptosomes (Redburn et al. 1976), but the exact cellular origin of these particles was unknown. A Ca^{++} -independent (^3H)-GABA release was also found in toad and goldfish horizontal cells which contain only very few or no synaptic vesicles (Schwartz 1982, Yazulla 1983). In this study on the intact rabbit retina with the label localized mainly to amacrine cells, potassium caused an increased release of (^3H)-GABA, which could not be inhibited by removing the Ca^{++} . This release was demonstrable only when the label was in neurons, not in glia. It would thus seem that nerve cells containing synaptic vesicles also have a mechanism for Ca^{++} -independent release of (^3H)-GABA.

Ouabain is a selective inhibitor of membrane ATP-ase, and therefore inhibits restoration of the ionic balance after a nerve impulse. Ouabain can increase the resting release of neurotransmitter in the absence of Ca^{++} and in the presence of EGTA (Vizi 1978), and has therefore been thought to show release of a cytoplasmic transmitter (O'Fallon et al. 1981, Yazulla & Kleinschmidt 1983). However, in these studies we find that ouabain will increase release both from neurons and glia and it is therefore not possible to use its effects as evidence for the presence of a release of (^3H)-GABA from neuronal cytoplasm. At present, the simplest explanation is that it is the result of an inhibition of the energy-dependent uptake mechanism into both glia and neurons.

In summary, the experiments have demonstrated that it is unlikely that there are any glutamate/aspartate or glycine receptors on the GABA neurons in the rabbit retina and it seems that there are no GABA autoreceptors on them. We have also found a calcium-independent, depolarization-induced release (^3H)-GABA from amacrine cells.

This study was supported by grants from the Helfrid and Lorentz Nilssons Stiftelse, the Thorsten and Elsa Segerfalks Stiftelse, the Carmen and Bertil Regnérs Stiftelse, the Anna Lisa and Sven-Eric Lundgrens Stiftelse and the Swedish Medical Research Council (project 14X-2321).

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GABA INHIBITS THE SPONTANEOUS RELEASE OF (³H)ACETYLCHOLINE
FROM THE CHICK RETINA

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SUMMARY

The release of (^3H)acetylcholine from the chick retina was studied. A 5mM increase in K^+ -concentration caused an increased release, which was Ca^{++} -dependent. The effect of 5mM K^+ was neither potentiated by bicuculline, nor inhibited by isoguvacine or muscimol. This indicates that the K^+ -induced release is not controlled by GABA. However, bicuculline and picrotoxin increased the spontaneous efflux of radioactivity, whereas GABA had no significant effect. The results indicate that cholinergic neurons are tonically inhibited by a continuous release of endogenous GABA. Neither glycine or strychnine, nor dopamine or haloperidol had any effect on the spontaneous release.

INTRODUCTION

Acetylcholine and gamma-aminobutyric acid (GABA) have both been characterized as neurotransmitters in the retina^{5,7}. The release of acetylcholine has been extensively studied in the rabbit retina^{6,8,9,10} and both the stimulus induced release (by light flashes) and the spontaneous release could be inhibited by GABA. The results indicate an interaction between cholinergic and GABAergic systems in the rabbit retina.

The aim of the present study was to examine the effects of GABA on the retinal acetylcholine release in a different species, the chick. Recently, GABA has been demonstrated to inhibit the release of acetylcholine from cholinergic neurons in culture¹. In this study, however, we used dissociated embryonic chick retinal neurons, and therefore, it was of interest to study GABA effects on acetylcholine release in the postnatal intact chick retina, and with a different technique.

MATERIALS AND METHODS

Ten-week-old chicken were injected with (³H)choline (20 uCi) intravitreally. One hour after the injection, the animals were decapitated, the eyes were enucleated, and the anterior segment and the vitreous were carefully removed. The posterior segment was everted and placed in a water jacketed superfusion chamber previously described in detail³. The retina was superfused in the dark at 37°C (1 ml/min) with a balanced salt solution², aerated with a gas mixture of 95% oxygen and 5% carbon dioxide, and left to equilibrate for 30 minutes before the start of the actual experiment.

In some experiments, the chicken were kept in the dark after the intravitreal injection and the dissection of the eye was performed in dim red light.

Potassium. In light adapted animals, the effects of K^+ (40mM, 20mM, 10mM and 5mM in addition to the normal 3.6mM in the superfusion solution) was studied for 2 minutes in a first series of experiments. In a second series, the retinae were superfused with a solution containing isoguvacine ($10^{-4}M$), muscimol ($10^{-4}M$), or bicuculline ($10^{-5}M$) for 8 min starting 6 min before 5 mM K^+ was added. In a third series, the effect of 5 mM K^+ was studied in a calcium deficient medium also containing EGTA ($10^{-4}M$).

Photic stimulation. The retinae of light adapted animals were equilibrated in the dark for 60 minutes in the superfusion chamber before the experiment started. In some of the experiments the superfusion solution contained eserine ($3 \times 10^{-4}M$), whereas in others no acetylcholine esterase inhibitor was added. The retinae were then stimulated with light flashes from a xenon flash lamp (3 Hz) for 10 minutes.

GABA, bicuculline, picrotoxin, glycine, strychnine, dopamine, and haloperidol. The retinae of dark adapted animals (1 h) were left to equilibrate for 30 minutes in darkness in the superfusion chamber before they were exposed to GABA ($10^{-3}M$), bicuculline ($10^{-5}M$), picrotoxin ($5 \times 10^{-5}M$), glycine ($10^{-3}M$), strychnine ($2 \times 10^{-5}M$), dopamine ($10^{-3}M$), or haloperidol ($2 \times 10^{-6}M$) for 15 minutes. In addition, the effect of GABA ($10^{-3}M$) was studied after 60 minutes of equilibration with a superfu-

sion solution containing eserine ($3 \times 10^{-4} \text{M}$).

All experiments were performed at least 4 times.

Superfusion techniques. One minute superfusate samples were collected (1 ml) and 500 μl of each sample was added to a commercial scintillation liquid. The radioactivity of the vials was registered in a scintillation spectrometer and quench corrections were measured with the external standard channels ratio method. The efflux curves were normalized and fitted to each other using a computer. The 5 minute period preceding the stimulation served as a reference for the normalization. Changes in the amount of radioactivity released were obtained by comparing the increase or decrease of radioactivity of the actual curve and that of a control curve at a given time using Mann-Whitney's two-tailed ranking test.

(^3H)Choline (80,0 Ci/mmol) was obtained from New England Nuclear Co and used as delivered. Isoguvacine was a gift from Dr Krogsgaard-Larsen, Copenhagen, Denmark. Muscimol was obtained from CIBA-Geigy, haloperidol from AB LEO, and the rest of the substances from Sigma Chemical Co.

RESULTS

Potassium. Potassium caused an increased release of radioactivity from the retina ($p < 0.01$) (table I). Between 40mM and 10mM K^+ , the effect was approximately the same. However, at a K^+ -concentration of 5mM. the effect on radioactive release from the retina was still evident, ($p < 0.01$) but significantly less than at 10mM K^+ ($p < 0.05$) (table I). The effect of 5mM K^+ is shown in fig 1. In a Ca^{++} -deficient medium, the releasing effect of 5mM K^+ was abolished ($p < 0.01$) (table I). When the

retinae were exposed to the GABA agonists, isoguvacine or muscimol, there was neither any change in the spontaneously released radioactivity, nor any influence on the increased release caused by 5mM K^+ . In addition, the GABA receptor blocker bicuculline did not change the release of radioactivity induced by 5mM K^+ (table I) (fig 2).

Photic stimulation. Light flashes did not influence the spontaneous release of radioactivity from the retina irrespective of the presence or absence of eserine in the superfusion medium.

GABA, bicuculline, picrotoxin, glycine, strychnine, dopamine, and haloperidol. GABA had no effect on the release of radioactivity from the retina, neither in the absence, nor in the presence of eserine in the superfusion medium. On the other hand, both bicuculline ($p < 0.01$) (table II) and picrotoxin ($p < 0.02$) (table II) increased the release of radioactivity, whereas glycine, strychnine, dopamine, and haloperidol did not change the release of radioactivity from the retina (table II). The time course changes induced by bicuculline and picrotoxin are shown in fig 3 and fig 4.

DISCUSSION

When the retina was exposed to K^+ (5mM in addition to the 3.6mM in the superfusion solution), there was a 12% increased release of radioactivity which was dependent on extracellular calcium. This agrees with the results of Baughman and Bader⁴ and indicates a release from a vesicular pool of neurotransmitter. In dealing with the chick retina, one has to be aware of the phenomenon called spreading depression^{11,13}. Potassium concentrations higher than 8mM cause reversible changes in the transparency of the retina but higher concentrations (at least 50mM) are required to elicit spreading depression¹². It thus seems unlikely that the release induced by a total K^+ -concentration of 8.6mM could be caused by such a mechanism.

In order to study the influence of GABA on the K^+ -induced release, the GABA receptor agonists, isoguvacine or muscimol, were present in the superfusion medium for 8 min starting 2 min before the stimulation with K^+ . None of these agents were able to depress the spontaneous release or the retinal response to K^+ . It may be that the cholinergic neurons are tonically inhibited by endogenous GABA, so that there are not enough GABA receptors available for the agonists to bind to.

The GABA antagonists, bicuculline and picrotoxin, increased the spontaneous release of radioactivity. This is in agreement with previous studies in the rabbit^{6,8,9,10} and further supports the assumption that the cholinergic neurons are controlled by endogenous GABA. Since blocking the GABA receptors with bicuculline did not increase the stimulus induced release (caused by 5mM K^+) above the background level caused by bicuculline itself (fig. 2), it thus seems that

GABA influences the spontaneous release of acetylcholine, but does not control the response to depolarization caused by K^+ .

In the present study, we monitored the release of radioactivity as an efficient measurement of the (3H)acetylcholine release. There is good evidence in the literature that this is a useful approach. When chicken retinæ preloaded with (3H)choline were exposed to K^+ , Baughman and Bader⁴ demonstrated that most of the radioactivity released consisted of (3H)acetylcholine. Similarly, Neal and Massey¹⁰ found, that the radioactivity released from the rabbit retina after light stimulation consisted mainly of (3H)acetylcholine. This was demonstrated also after the exposure to bicuculline or picrotoxin. In these studies an inhibitor of cholinesterase was present. In the present study, such an inhibitor was not found necessary. When an increased release failed to appear, most of the experiments were repeated with eserine added to the superfusion solution, but still no increase in the release could be detected. Thus, it is not likely that a negative result was due to a reuptake of (3H)choline.

Previously, xenon light flashes have been demonstrated to cause a release of (3H)acetylcholine from the rabbit retina in vivo^{6,8,9}. We have not been able to repeat these results in the chick retina in vitro which could be explained by species differences. However, the experiments were repeated in the rabbit retina in vitro (unpublished data) and still no increased release of radioactivity was observed. This is in agreement with results obtained by Neal (1981, personal communication) and thus the most plausible explanation for the inability to demonstrate a light induced

release is the use of different experimental techniques.

Glycine, strychnine, dopamine, and haloperidol were all unable to change the spontaneous efflux of radioactivity. Cunningham and Neal⁶ and Massey and Redburn⁹, were similarly unable to demonstrate any effect of strychnine or dopamine on the resting release of (³H)acetylcholine in the rabbit retina.

GABA receptors have been demonstrated previously on embryonic cholinergic neurons in the chick¹. The present study indicates that cholinergic neurons in the chick retina are tonically inhibited by endogenous GABA suggesting a direct interaction between GABAergic and cholinergic neurons also in the postnatal intact chick retina.

Acknowledgements

This study was supported by grants from the Swedish Society for Medical Research, the Ebba Henrikssons Stiftelse, the University of Lund, and the Swedish Medical Research Council (project 14X-2321).

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TABLE I

K^+ -induced release of radioactivity from chicken retinae preloaded with (3H)acetylcholine and the effects of Ca^{++} -free medium, bicuculline, isoguvacine, and muscimol on this release. n=number of experiments; MV=mean value; p indicates the statistical significance calculated with Mann-Whitney's two-tailed ranking test when compared with the effect of 5mM K^+ . Therefore, range rather than standard errors of the mean is presented in this table.

	% increased release			
	n	range	MV	p
5mM K^+	6	0 to 38	12	
10mM K^+	6	13 to 49	30	<0.05
Ca^{++} free medium + 0.1mM EGTA + 5mM K^+	6	(-6)to 0	-3	<0.01
bicuculline ($10^{-5}M$) + 5mM K^+	6	(-6)to 56	14	N.S.
muscimol ($10^{-4}M$) + 5mM K^+	5	5 to 76	35	N.S.
isoguvacine ($10^{-4}M$) + 5mM K^+	4	0 to 58	25	N.S.

TABLE II

Release of radioactivity from chicken retinae preloaded with (^3H)acetylcholine 6 min after the exposure to GABA, glycine, dopamine, or to some agonists or antagonists. n=number of experiments; MV=mean value; p indicates the statistical significance calculated by Mann-Whitney's two-tailed ranking test when compared with the spontaneous release. Therefore, range rather than standard error of the mean is presented in this table.

	% increased release			
	n	range	MV	p
Spontaneous efflux	5	(-23) to (-3)	(-12)	
GABA (10^{-3}M)	4	(-11) to (4)	(-4)	N.S.
bicuculline (10^{-5}M)	8	(-12) to 25	7	<0.01
picrotoxin ($5 \times 10^{-5}\text{M}$)	8	(-9) to 37	12	<0.02
glycine (10^{-3}M)	5	(-8) to 3	(-3)	<0.1
strychnine ($2 \times 10^{-5}\text{M}$)	4	(-5) to 9	1	N.S.
dopamine (10^{-3}M)	5	(-18) to (-7)	(-13)	N.S.
haloperidol ($2 \times 10^{-6}\text{M}$)	5	(-33) to 0	(-11)	N.S.



Fig. 1. Chicken retinæ exposed to (^3H)acetylcholine and the releasing effect of 5mM K^+ . SEM are indicated by vertical bars: 6 experiments. The dotted line represents the spontaneous efflux of (^3H)acetylcholine.



Fig. 2. Chicken retinæ preloaded with (^3H)acetylcholine and the releasing effect of 5mM K^+ 6 min after the exposure to bicuculline (10^{-5}M). SEM are indicated by vertical bars: 6 experiments. The dotted line represents the effect of 5mM K^+ in the absence of bicuculline.

Relative
radioactivity

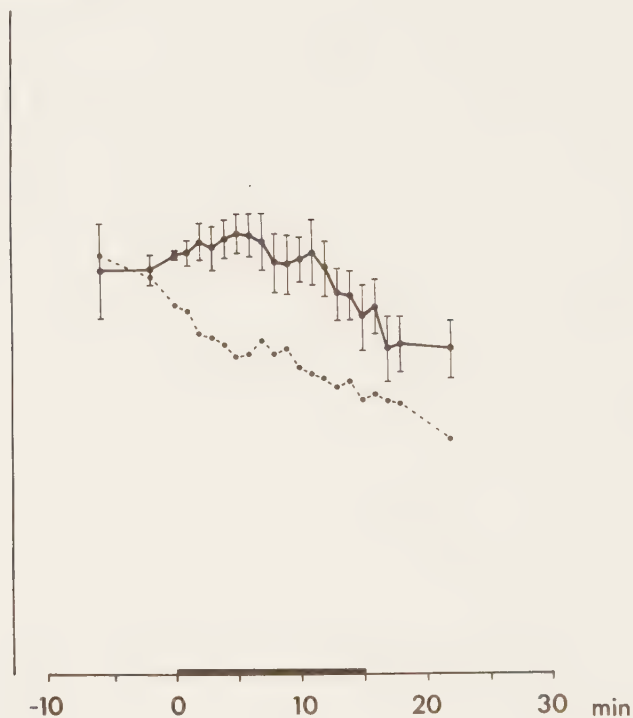


Fig. 3. Chicken retinæ exposed to (^3H)acetylcholine and the releasing effect of bicuculline (10^{-5}M). SEM are indicated by vertical bars: 8 experiments. The dotted line represents the spontaneous efflux of radioactivity.

Relative
radioactivity

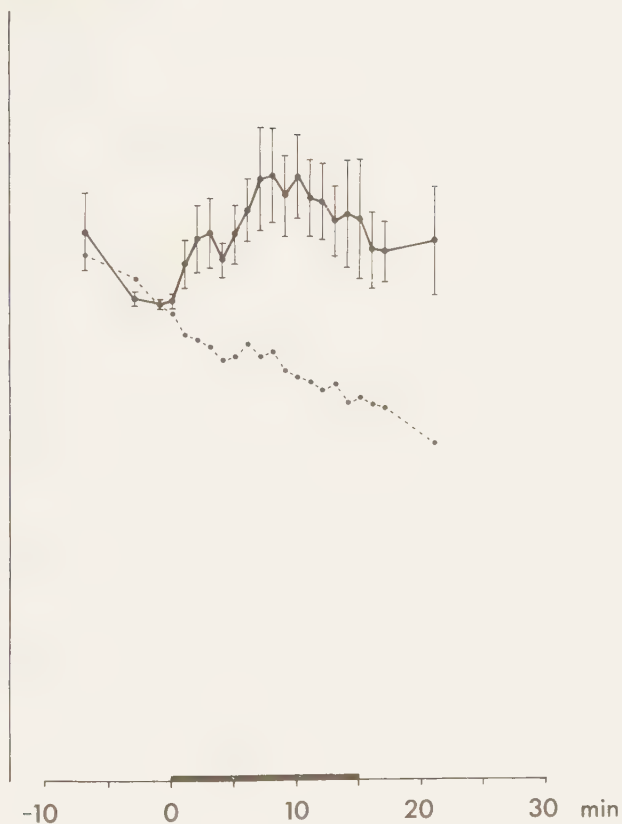


Fig. 4. Chicken retinæ exposed to acetylcholine and the releasing effect of picrotoxin ($5 \times 10^{-5} \text{M}$). SEM are indicated by vertical bars: 8 experiments. The dotted line represents the spontaneous efflux of radioactivity.

Brain Research. in press 1984

GABA-MEDIATED INHIBITION AT CHOLINERGIC SYNAPSES FORMED BY
CULTURED RETINAL NEURONS

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SUMMARY

The purpose of this study was to investigate the effect of GABA on the function of synapses formed by cholinergic neurons derived from the chick retina. We used an experimental culture system in which striated muscle cells served as postsynaptic targets for cholinergic neurons of the retina. This cell culture system permitted the physiological monitoring of acetylcholine release at synapses formed by retinal neurons. By plating a low density of dissociated retinal cells with myotubes, it was possible to study relatively isolated, presynaptic cholinergic neurons. We found that GABA and its agonists, muscimol and isoguvacine, inhibited spontaneous transmission at retina-muscle synapses. These inhibitory effects were reversibly blocked by bicuculline, a GABA receptor antagonist. The benzodiazepine, flurazepam, potentiated GABA-mediated inhibition. Overall, our findings suggest a direct inhibitory action of GABA on the cholinergic retinal neurons studied in our cell culture system.

GABAergic and cholinergic systems of the vertebrate retina interact^{3,13,14,16,20}. For example, GABA inhibits the release of acetylcholine from the retina^{13,14,16}. However, it has not been possible from studies of the intact retina to determine whether GABA-mediated inhibition of cholinergic activity is a monosynaptic or a polysynaptic effect. In this study, we considered the possibility that a cell culture system may facilitate physiological and pharmacological studies of GABAergic effects on cholinergic retinal neurons. Our results indicate that GABA exerts direct inhibitory effects on the release of acetylcholine from cholinergic retinal neurons.

We used a culture system which permits the monitoring of acetylcholine release at synapses formed by neurons derived from the chick retina. In our culture system, striated muscle cells serve as postsynaptic targets for cholinergic retinal neurons. By recording postsynaptic responses, we demonstrated previously that embryonic retinal neurons can form functional synapses in culture with myotubes^{22,23}. Muscle cells served as useful as postsynaptic targets since their membranes have areas with a high density of cholinergic receptors^{5,11} and because their response to acetylcholine has been well studied both in vivo¹⁰ and in culture⁶. Importantly, their relatively large size permits prolonged intracellular monitoring of postsynaptic responses. With our culture system, it is possible, therefore, to detect the release of acetylcholine from a single retinal neuron^{19,23}. Here, we report a direct inhibitory action of GABA on cholinergic transmission at retina-muscle synapses.

MATERIALS AND METHODS

The methods for the preparation and maintenance of retina-muscle cell cultures as well as for many of the electrophysiological assays have been presented in detail previously^{19,21,27}. Myoblasts were obtained from the hind limbs of postnatal day 1 rats, dissociated with trypsin, cultured in 35 mm dishes and allowed to fuse into myotubes 200 to 300 μ m in length. Trypsin-dissociated retinal cells from staged chick embryos⁷ were harvested, added (0.05 to 10×10^6 cells per dish) to 5 to 7 day old muscle culture, incubated in medium A which consisted of 90% basal medium of Eagle with Earle's salts (GIBCO) and 10% fetal bovine serum (MA Bioproducts). During the electrophysiological experiments, the cultures were maintained in medium A at pH 7.4 and kept at $32^\circ \pm 0.50$. Intracellular recordings from myotubes were made with 3M KCl-filled micropipets which had tip resistances of 30 to 60 M. After amplification in the conventional manner, potentials were displayed on an oscilloscope (Tektronics 5400) and penwriter (Gould 260). On channel of the penwriter received an unfiltered signal which permitted continuous monitoring of the membrane potential. Another channel displayed a high gain, AC-coupled signal which was used to quantitate the synaptic events. The noise level of the recordings was between 200 to 400 μ V.

GABA, isoguvacine, and muscimol were applied near retinal neurons by microiontophoresis (≤ 80 nA) from single barrel micropipets (tip resistances 5 to 15 M) filled with 0.5M or, in the case of muscimol 0.1M, of the chemical at pH 4. Bicuculline (0.5M, pH 4) and flurazepam (40mM, pH 3.6) were also applied by microiontophoresis (≤ 50 nA). Isoguvacine was a

gift of Dr. P. Krogsgaard-Larsen. Flurazepam (Hoffman LaRoche) was provided by Dr. B.D. Waterhouse. The other chemicals were obtained from Sigma.

Muscle cells innervated by retinal neurons were detected by the presence of spontaneous synaptic potentials as described previously²². When an innervated muscle cell was found, the general location of the presynaptic neuron was determined as detailed elsewhere^{21,27}. In brief, glutamate (0.5M, pH 8) was applied by microiontophoresis (≤ 55 nA, ≤ 1 min) from a micropipet (tip resistance 10-25 M) systematically along the entire length of the innervated myotube in search of the site where glutamate could evoke synaptic input. As reported earlier²³, a localized site (approximately 40 μ m in diameter) where glutamate application evokes synaptic input can be found for 95% of the innervated muscle cells examined 36 h after the addition of embryonic day 10 retinal cells or 48 h after the addition of embryonic day 8 cells. In all cases in which evoked synaptic input was detected, the glutamate-filled micropipet had been positioned near retinal neurons which were positioned on or adjacent to the muscle cell being examined. In experiments using a high density of retinal cells (e.g. 10^7 cells/35 mm dish), numerous retinal cells were located near the site of glutamate application.

In cocultures containing a relatively low density of retinal cells (5×10^4 cells/35 mm dish), a specific retinal cell could be identified as the putative presynaptic neuron at greater than 90% of the innervated myotubes chosen for study (see reference 23 for further details). Importantly, for the localization of presynaptic neurons in the low density cultures, glutamate was applied very focally by pass-

ing brief (≤ 10 sec) pulses of current (≤ 25 nA) through glutamate-filled micropipets of high resistance (55–70 M Ω). Localization was attempted when an innervated myotube had four or less retinal cells on or adjacent to it and the retinal cells were separated by at least 40 μ m. In our previous study, zero in 91 postsynaptic muscle cells were found to be innervated by more than one retinal neuron²³. The identification of a presynaptic neuron could be further substantiated by observing that irreversible damage to the selected neuron (e.g., following unsuccessful attempts to intracellular recording) or physical dislocation of the nerve cell from the myotube was associated invariably with a prompt termination of postsynaptic activity²³.

Once an input site was identified, glutamate application was terminated and spontaneous synaptic activity was allowed to return to control levels. Then, the effect of GABA or its agonists on the rate of spontaneous synaptic input to the muscle cell was assayed. If the rate of synaptic potential was reduced by at least 33% during the time of drug application, the effect was declared as being inhibitory. No attempt was made to focally apply GABA onto only specific areas of the presynaptic neuron. The GABA-filled micropipets were typically positioned 20 to 30 μ m from the soma of the neuron.

In the preparation for scanning electron microscopy, culture dishes were washed ten times at room temperature with phosphate buffered saline pH 7.2. The cultures were then fixed for one half hour at room temperature in 2% glutaraldehyde and 1% paraformaldehyde in 0.13M sodium cacodylate buffer, pH 7.2. Samples were kept in fixative at 4°C overnight, then, dehydrated in ascending concentrations of ethanol. The

dishes were broken into 3 to 5 small pieces for critical-point-drying with CO_2 . They were then mounted on aluminium stubs and sputter coated with 200 to 300 Å layer of gold-palladium²⁶. Scanning electron microscopy was performed using a Hitachi S430 microscope at 15 kV accelerating voltage.

RESULTS

Inhibition of cholinergic transmission

In the initial series of experiments, retinal cells (10×10^6) dissociated from day 10 embryos were added to muscle cultures. The effects of GABA and its agonists, muscimol² and isoguvacine¹², were assessed after 36 h of coculture. Inhibitory effects on spontaneous transmission were detected during application of GABA or its agonists as illustrated in Figure 1. In 81% (35/43) of the innervated muscle cells examined, GABA could be demonstrated to have an inhibitory effect on spontaneous synaptic activity. Inhibition by muscimol or isoguvacine was found in 78% (7/9) and 74% (14/19), respectively, of the innervated myotubes studied. Importantly, control experiments showed that neither GABA, muscimol nor isoguvacine when applied by microiontophoresis altered the depolarizing response of muscle cells to microiontophoretically applied acetylcholine nor induced changes in the membrane potential of myotubes. Currents passed through micropipets similar to those used for GABA or its agonists, but filled with 0.5M NaCl at pH 4, did not affect synaptic activity in myotubes.

In another series of experiments, a relatively low number of retinal cells (5×10^4) dissociated from day 8 embryos was cocultured with muscle cells for 48 h. In this way, individual neurons could be studied in relative isolation

from other retinal cells^{19,23}. In cocultures sparsely populated by retinal cells, it was possible to identify retinal neurons that were presynaptic to muscle cells (Figure 2). In these low density cocultures, 91% (10/11) of the innervated muscle cells examined could be demonstrated to have their synaptic input inhibited by the application of GABA onto the presynaptic retinal neurons.

Mediation by receptors

It was important to determine whether GABA exerted its inhibitory effect by activating GABA receptors. Pharmacological tests of receptor specificity demonstrated that the observed GABA-mediated inhibitory effect could be reversibly blocked by bicuculline, a relatively specific GABA receptor antagonist³. An example of a bicuculline-induced blockade of an inhibitory response to GABA is shown in Figure 3. During the application of bicuculline, GABA-induced inhibition of synaptic input to this innervated myotube was abolished almost completely. After cessation of bicuculline-exposure, the inhibitory effect was again detected. Similar reversible antagonism of the GABA-mediated effect was observed in 75% (6/8) of the innervated muscle cells examined. The inhibitory effects of isoguvacine and muscimol were similarly reversed by bicuculline.

Potentiation by a benzodiazepine

Since benzodiazepine receptors have been found in the vertebrate retina^{1,4,9,15,18,24,25,28} and are presumed to be associated closely with GABA receptors¹⁷, it was of interest to examine the effect of a benzodiazepine on the GABA-

mediated inhibition of transmission at retina-muscle synapses. Figure 4 shows an experiment using flurazepam, a water soluble benzodiazepine. Prior to exposure to flurazepam, a relatively weak inhibitory effect of GABA on spontaneous synaptic transmission was observed. However, following application of the benzodiazepine, a profound inhibitory response to GABA was observed. This effect of flurazepam was relatively long lasting, yet clearly reversible (Figure 4). Control experiments revealed that the microiontophoretic application of flurazepam did not affect the resting membrane potential of myotubes nor alter the responses of muscle cells to microiontophoretically applied acetylcholine.

DISCUSSION

The results show that GABA inhibits cholinergic transmission at synapses formed in culture by neurons derived from the chick retina. The GABA agonists, muscimol and isoguvacine, also mediated similar inhibitory effects on spontaneous activity at retina-muscle synapses. These inhibitory effects of GABA and its agonists were blocked reversibly by the GABA receptor antagonist, bicuculline. In control experiments, GABA was not found to influence the membrane potential of muscle cells nor the responses of myotubes to microiontophoretically applied acetylcholine. Taken together, these findings indicate that GABA mediates its effect on cholinergic synaptic transmission via bicuculline-sensitive GABA receptors located on retinal neurons.

However, from experiments using a high density of dissociated neurons, it was not clear whether GABA inhibited synaptic input to muscle cells by directly or indirectly (i.e. via polysynaptic pathways) affecting the presynaptic cholinergic neurons. To address this issue, a relatively low density of retinal neurons was added to muscle cell cultures. This permitted an analysis of GABA-mediated inhibition of synaptic input to myotubes innervated by a single cholinergic neuron (see reference 23). Despite the relative isolation of these presynaptic neurons from other retinal cells, GABA-induced inhibition could be demonstrated at more than 90% of the retina-muscle synapses. This finding suggests that GABA directly affects the presynaptic cholinergic neurons.

This study also demonstrates that the inhibitory effect of GABA on cholinergic transmission at retina-muscle synapses can be potentiated by a benzodiazepine. Although there are a

considerable number of studies showing the presence of benzodiazepine receptors in the vertebrate retina^{1,4,9,15,24,25,28}, the findings reported here are the first evidence that benzodiazepine-GABA interactions can influence information processing by retinal neurons. Our observations that benzodiazepines act by potentiating inhibition mediated by bicuculline-sensitive GABAergic receptors are in general agreement with studies of other parts of the central nervous system¹⁷. An implication of the benzodiazepine-GABA interactions may be that the functional potency of the GABAergic systems in retina is dynamic, not static.

A general aim of this study was to assess the possibility that a culture system may be useful in physiological and pharmacological studies of GABAergic effects on cholinergic retinal neurons. The retina-muscle-system does appear to be helpful. For example, the finding that inhibition by GABA was demonstrated at 91% of the retina-muscle synapses in the low density cocultures indicates that the cholinergic neurons studied in this system are a relatively homogeneous population with respect to their responsiveness to GABA. The ability to examine the cholinergic retinal neurons which innervate myotubes in culture should be of great experimental value. By applying the technique of whole cell recording⁸ to the study of relatively isolated neurons, the direct inhibitory effect of GABA can be explored further. Also, the techniques of patch-clamping should allow the study of ion channels that may be linked to GABA receptors. Finally, the observation that GABA-mediated inhibition can be potentiated in a reversible manner by flurazepam provides the opportunity to systematically examine the effects of benzodiazepine-GABA interactions on the function of cholinergic retinal neurons.

Acknowledgements: We thank Dr. W.P. Wergin for use of his scanning electron microscope, Dr. P. Krogsgaard-Larsen for providing isoguvacine and Dr. B.D. Waterhouse for giving flurazepam.

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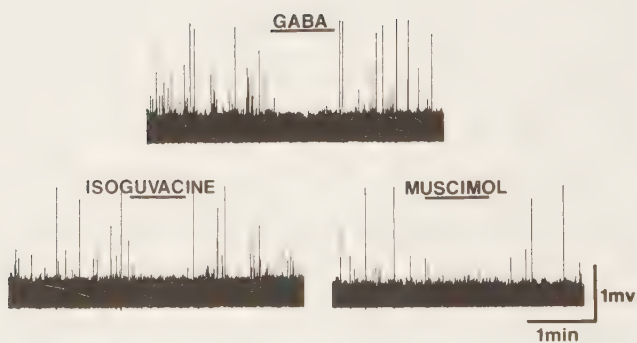


Figure 1. Effects of GABA, isoguvacine and muscimol on the spontaneous postsynaptic responses of innervated muscle cells. Transient depolarizations of the muscle cells were detected with an intracellular micropipet and are shown in the penwriter recordings. The bars above the penwriter records indicate the times for the microiontophoretic application (55nA) of GABA or its agonists.



Figure 2. Scanning electron photomicrograph of an innervated muscle cell (short arrows) with its putative presynaptic cholinergic retinal neuron (long arrow). Retinal cells derived from a day 8 chick embryo had been in the culture with myotubes for 48 hr. See the materials and methods section for details concerning the procedure for localizing the presynaptic neuron. Bar shows 20 μ m.

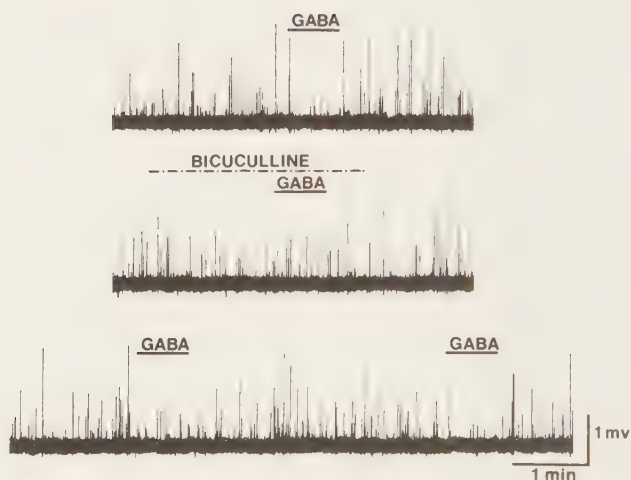


Figure 3. Effect of bicuculline on GABA-mediated inhibition. The penwriter records show the synaptic activity of a myotube cocultured for 36 h with retinal neurons from 10 day old chick embryos. The upper panel demonstrates that the microiontophoretic application (20 nA) of GABA onto retinal neurons located on or near this muscle cell could inhibit the synaptic activity. In the presence of bicuculline (40 nA), this inhibitory effect of GABA was blocked (middle panel). The lower panel shows that the effect of bicuculline was reversible. Bars indicate the times for the microiontophoresis of GABA (solid bar) or bicuculline (broken bar).

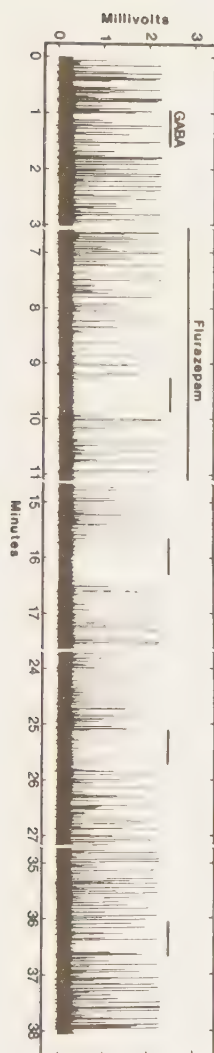


Figure 4. Effect of flurazepam on GABA-mediated inhibition of synaptic transmission. The penwriter record shows the postsynaptic activity of a muscle cell 36 h after the addition of embryonic day 10 retinal neurons. The bars show the time(s) for the microiontophoresis of GABA (22 nA) and flurazepam (5 nA).

